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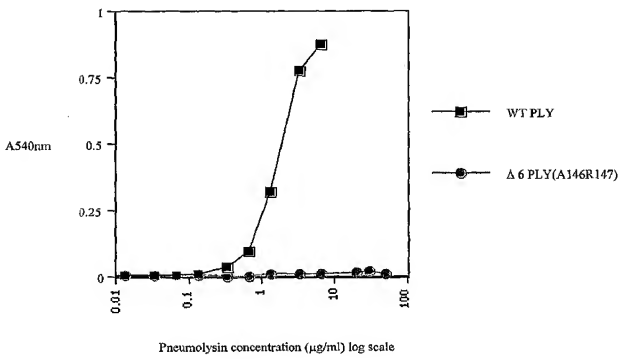
(54) Title: MUTANT CHOLESTEROL-BINDING CYTOLYSIN PROTEINS



(57) Abstract: The invention relates to immunogenic compositions comprising mutant cytolysin proteins. The invention further relates to such proteins and nucleic acids encoding these proteins. In particular embodiments, the invention is directed to an isolated mutant cytolysin protein, wherein the mutant protein differs from the wild type protein by the deletion of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence. In certain embodiments, the mutant protein differs from the wild type protein by the deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence. In particular embodiments, the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

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MUTANT CHOLESTEROL-BINDING CYTOLYSIN PROTEINS

Field of the Invention

The present invention relates to immunogenic compositions comprising mutant cholesterol-binding cytolysin proteins. The invention further relates to such proteins and nucleic acids encoding these proteins.

Background of the Invention

Streptococcus pneumoniae is an important pathogen, causing invasive diseases such as pneumonia, meningitis and bacteraemia. Even in regions where effective antibiotic therapy is freely available, the mortality rate from pneumococcal pneumonia can be as high as 19% in hospitalised patients. In developing countries, in excess of 3 million children under the age of 5 years die each year from pneumonia, of which *S. pneumoniae* is the commonest causative agent. *S. pneumoniae* also causes less serious, but highly prevalent infections such as otitis media and sinusitis, which have a significant impact on health-care costs in developed countries. Otitis media is especially important in young children, while sinusitis affects both children and adults.

The heptavalent polysaccharide conjugate vaccine from Wyeth, sold as Prevnar® in the United States and as Prevenar® in the rest of the world, is currently the only effective conjugate vaccine available for protection against *Streptococcus pneumoniae* infection (Kyaw et al, 2002; Hausdorff et al, 2000). The vaccine comprises seven purified *Streptococcus* capsular polysaccharides (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) out of a possible 90 (Kalin, 1998), each conjugated to a carrier protein. Preparation of such a vaccine is described in US Patent 4,673,574 (Anderson). The protein used for conjugation of the capsular polysaccharides is a diphtheria toxoid, CRM_{197}, offering an increase in the immunogenicity of the vaccine in infants (Blum et al, 2000; Katkocin, 2000). However, each serotype of *S. pneumoniae* has a

structurally distinct capsular polysaccharide, such that immunization with one serotype tends not to confer protection against the majority of the other serotypes, although some cross-protection does occur against vaccine-related serotypes (Whitney et al., 2003).

Complementary approaches to serotype-specific immunization are being investigated. A possibility is to also use a species-common virulence factor such as Pneumolysin (PLY), the 53kDa toxin produced by all invasive strains of *S. pneumoniae* (Paton et al, 1993). PLY could be used alone or as a carrier protein conjugated to the polysaccharides in Prevnar®, offering increased efficacy. Alexander et al (1994) demonstrated that immunisation of mice with a PLY toxoid conferred immune protection upon challenge with 9 different serotypes of *S. pneumoniae*. PLY has been shown to stimulate an immune response similar to that of *S. pneumoniae* infection by activating the classical complement pathway (Paton et al, 1984) and inducing apoptosis of neutrophils and macrophages (Cockeran et al, 2002; Kadioglu et al, 2000).

PLY belongs to the group of Cholesterol-binding Cytolysins (CBCs) that bind to the cholesterol of host cell membranes prior to formation of large 30-50mer ring structures that create lytic pores (Palmer, 2001; Jedrzejas, 2001). The mechanism of pore-formation is not fully understood and there is much debate over the sequence of events (Bonev et al, 2000; Shepard et al, 1998). However, the ability to form pores means that native PLY is highly toxic, which is a problem in terms of the development of immunogenic compositions.

Although the conjugation process used in production would render PLY non-toxic, it would be more favourable to start with a non-toxic form. Further, a toxic form would be difficult to use in preparation of unconjugated immunogenic compositions. The toxicity of PLY can be significantly reduced by site-directed mutagenesis to create PLY toxoids, known as Pneumolysoids (Paton, 1996).

A variety of such toxoids exist and have been shown to give immune protection, either independently or when conjugated to polysaccharides, to mice in response to a challenge with virulent type 2 D39 *S. pneumoniae* (Paton et al, 1991; Alexander et al, 1994). Most mutations have previously been created in the highly conserved 11 amino acid region near the C' terminus (Mitchell et al, 1992; Berry et al, 1995). This site has been shown to be involved in binding to the host cell (de los Toyos et al, 1996). A number of such mutated forms of PLY are described in International Patent Application WO 90/06951; each of the mutations described in this publication is towards the C' terminus of the protein.

Generation of a mutant form of perfringolysin (PFO), another CBC, is described in Hotze et al (2002). The mutant PFO has a Y_{181} to A_{181} mutation. The mutant protein apparently has a reduced pore formation activity when compared with the non-mutant form of PFO. However, there is no teaching of any possible uses for this mutant, and no teaching or suggestion that corresponding mutations in other CBC proteins may result in similar functional changes.

A further problem with PLY is that it aggregates upon large-scale production, a problem which must be solved in order for PLY to be used in immunogenic compositions. It is believed that the aggregation of PLY is related to the oligomerisation of PLY involved in pore formation. The present invention thus attempts to reduce or eliminate PLY-PLY interaction (oligomerisation), such that the chance of aggregation during large-scale production will decrease, thereby creating an easily purified form of PLY.

Toyos et al (1996) describe the raising of monoclonal antibodies (mAbs) to various regions of PLY, and probing of the whole toxin and a 'proteinase K nicked' form. Proteinase K cuts PLY into a 37kDa and 15kDa fragment. Antibody mAb PLY 4 only recognised whole PLY, and neither of the fragments, indicating that the epitope on PLY for this mAb is within the nicked region. When PLY was pre-incubated with mAb PLY 4, then

added to liposomes, the toxin no longer formed pores on the liposome membrane. This implies that the site blocked by mAb PLY 4 (thought to be the Asparagine N_{143} region) is the site responsible for interaction with other PLY monomers to form oligomeric pores. Oligomerisation of Streptolysin O from *Streptococcus sp.* can also be blocked by mAbs as demonstrated by Hugo et al in 1986. It is unknown whether the antibodies directly prevent oligomerisation by binding to the oligomerisation site or if there is an association that sterically hinders the interaction of toxin monomers.

Monoclonal antibody PLY 4 has been further characterised by Suárez-Álvarez et al (2003) and they suggest that the epitope for mAb PLY 4 is further downstream than the N_{143} region initially proposed by Toyos et al in 1996. The site of recognition now appears to be conformation dependent and within amino acids E_{151} - Y_{247} and not within the N_{143} region.

Previously a $N_{142}N_{143}$ deletion and $N_{143}D$ substitution within PLY were created by the present inventors as initial steps to understanding this region and its role in oligomerisation. Characterisation of both mutants revealed identical behaviour to native PLY in terms of haemolysis and pore formation (Search, 2002), suggesting that oligomerisation was not blocked, and the toxicity of the mutants remained unchanged. Thus, previously created mutant PLY forms do not exhibit reduced toxicity or reduced oligomerisation, suggesting that these mutations will not be of assistance in the production of immunogenic compositions.

Summary of the Invention

The present invention relates broadly to immunogenic compositions comprising mutant bacterial cytolysin proteins. The invention further relates to such proteins and nucleic acids encoding these proteins.

Thus, in one aspect, the invention is directed to an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein

by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, such that the toxicity of the mutant cytolysin is reduced relative to that of the wild-type cytolysin protein. The mutation may be located within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin (PLY) sequence.

Where the mutant cytolysin is a mutant perfringolysin comprising a substitution or deletion at Y_{181}, and in particular, a Y_{181} to A_{181} substitution, the mutant comprises a further mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, wherein the further mutation is capable in isolation of reducing the toxicity of the wild type sequence. Thus the toxicity of the mutant will be lower than that of a perfringolysin mutant having only the mutation at position 181 of wild type perfringolysin.

The mutation may be a deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence.

The mutant PLY protein may differ from the wild type protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin (PLY) sequence. In some embodiments the mutant cytolysin is not a mutant perfringolysin having a substitution at position Y_{181}.

For example, the mutant cytolysin protein may differ from the wild type protein by the substitution or deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin (PLY) sequence, as exemplified by the deletion of amino acids corresponding to valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

Any of the foregoing mutant cytolysin proteins may further comprise at least one amino acid substitution or

deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

The isolated mutant cytolysin protein has reduced toxicity to mammals. This is typically a consequence of having reduced pore-forming activity, which may be associated with reduced haemolytic activity and/or reduced oligomerisation activity, as compared with wild type protein. Desirably, although not necessarily, the mutant cytolysin protein has reduced oligomerisation activity to facilitate purification and subsequent manipulation.

In a further aspect, the invention is directed to an immunogenic conjugate comprising: (a) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and (b) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein. The mutation may be located within the region of amino acids corresponding to 144 to 151 of the wild type pneumolysin sequence.

The mutation may be a substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

The mutant cytolysin protein of the immunogenic conjugate may differ from the wild type cytolysin protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

For example, the mutant cytolysin protein of the immunogenic conjugate differs from the wild type cytolysin protein by the substitution or deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence, as exemplified by

the deletion of amino acids corresponding to valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151 of the wild type pneumolysin sequence.

Any of the foregoing mutant cytolysin proteins of the immunogenic conjugate may further comprise at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

The saccharide, oligosaccharide or polysaccharide of the immunogenic conjugate may be bacterial in origin, and may be derived from the same species, e.g. the same strain, as the cytolysin.

In a further aspect, the invention provides an isolated and purified nucleic acid sequence comprising a nucleic acid sequence a) encoding a mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin is reduced relative to that of the wild-type cytolysin protein; or b) which is complementary to a nucleic acid sequence defined in a).

Where the mutant cytolysin is a mutant perfringolysin comprising a substitution or deletion at Y_{181}, and in particular, a Y_{181} to A_{181} substitution, the mutant comprises a further mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, wherein the further mutation is capable in isolation of reducing the toxicity of the wild type sequence. Thus the toxicity of the mutant will be lower than that of a perfringolysin mutant having only the mutation at position 181 of wild type perfringolysin.

The mutation may be located within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

The mutation may be a substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

The nucleic acid sequence may encode a mutant cytolysin protein which differs from the wild type protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

For example, the nucleic acid sequence may encode a mutant cytolysin protein which differs from the wild type protein by the substitution or deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence, as exemplified by the substitution or deletion of amino acids corresponding to valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151 of the pneumolysin sequence.

The mutant cytolysin proteins encoded by the nucleic acid sequence may be any of the foregoing, and may further comprise at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

In a still further aspect, the invention provides a recombinant expression vector which comprises any of the foregoing isolated and purified nucleic acid sequences encoding a mutant cytolysin protein, as well as a recombinant host cell transformed, transfected or infected with such a recombinant expression vector.

In another aspect, the invention provides a method of producing an isolated mutant cytolysin protein of the invention, wherein the mutant cytolysin protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type cytolysin protein, the method comprising: a) transforming, transfecting

or infecting a host cell with a recombinant expression vector as described above and culturing the host cell under conditions which permit the expression of said mutant cytolysin protein by the host cell; and b) recovering the mutant cytolysin protein from the culture.

In still another aspect, there is provided an immunogenic composition which comprises: a) an isolated mutant cytolysin protein, in unconjugated form or as part of an immunogenic conjugate as described above, wherein the mutant cytolysin protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type cytolysin protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

The mutation may be located within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

The mutation may be a substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

The isolated mutant cytolysin protein of the composition may differ from the wild type cytolysin protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

For example, the mutant cytolysin protein may differ from the wild type protein by the substitution or deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence, as exemplified by the substitution or deletion of amino acids corresponding to valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151 of the pneumolysin sequence.

The immunogenic composition may contain any of the foregoing mutant cytolysin proteins, in unconjugated form or

as part of an immunogenic conjugate as described above, which further comprises at least one amino acid substitution or deletion in at least one of the regions of amino acids corresponding to 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

Thus, the immunogenic composition may comprise: a) an immunogenic conjugate comprising: (i) a saccharide, oligosaccharide or polysaccharide, which may be bacterial, and may be derived from the same species, e.g. the same strain, as the cytolysin; and (ii) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type cytolysin protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier. The composition may comprise saccharides, oligosaccharides or polysaccharides from a plurality of bacterial species and/or strains.

In further aspects, the invention is directed to a method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition which comprises: a) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type cytolysin protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

The immunogenic composition may comprise: a) an immunogenic conjugate comprising: (i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and (ii) an isolated mutant cytolysin protein as described herein.

In another aspect, the invention is directed to the use of any of the isolated mutant cytolysin proteins or immunogenic conjugates of the invention in the preparation of an immunogenic composition. The invention also provides a method of preparing an immunogenic composition, comprising the step of admixing a mutant protein or immunogenic conjugate of the invention with a pharmaceutically acceptable carrier.

The immunogenic compositions of the invention may be used for the prophylaxis or treatment of bacterial infection. In particular they are useful for the prophylaxis or treatment of infection by the bacteria from which the cytolysins are derived. However they may also be useful for prophylaxis or treatment of infections of bacteria having cholesterol-binding cytolysins, and particularly those which are immunologically cross reactive with the cytolyin of the composition; that is to say, which are capable of being bound by antibodies which will bind to that cytolysin.

In a further aspect, the present invention provides an isolated mutant cytolysin protein or immunogenic conjugate of the invention for use in a method of medical treatment.

In a still further aspect, the invention is directed to a method of preparation of an immunogenic composition, the method comprising the steps of: providing an isolated mutant cytolysin protein as described herein; and conjugating the mutant protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein. The mutant protein in the foregoing method may be conjugated to a polysaccharide derived from the same species as the cytolysin. The method may comprise the further step of admixing the conjugate thus obtained with a pharmaceutically acceptable carrier.

In a further aspect, the invention is directed to a method of screening candidate mutant cytolysin proteins for suitability for use in immunogenic compositions, the method comprising the steps of: providing a mutant cytolysin protein; testing the mutant protein for haemolytic activity;

testing the mutant cytolysin protein for oligomerisation activity; and comparing the haemolytic and oligomerisation activity of the mutant cytolysin protein with those of a non-mutant protein, e.g. a wild type protein.

In all aspects of the invention, the cytolysin may be from any suitable species. Examples of cytolysins and the species in which they are found are Pneumolysin from *Streptococcus pneumoniae*; Perfringolysin O from *Clostridium perfringens*; Intermedilysin from *Streptococcus intermedius*; Alveolysin from *Bacillus alvei*; Anthrolysin from *Bacillus anthracis*; Putative Cereolysin from *Bacillus cereus*; Ivanolysin O from *Listeria ivanovii*; Pyolysin from *Arcanobacterium pyogenes*; Seeligeriolysin O from *Listeria seeligeri*; Streptolysin O from *S. pyogenes*; Suilysin from *Streptococcus suis*; Tetanolysin from *Clostridium tetani*; Listeriolysin O from *Listeria monocytogenes*, Streptolysin O from *Streptococcus equisimilis*, Streptolysin O from *S. canis*, Thuringiolysin O from *Bacillus thuringiensis*, Latersporolysin O from *B. laterosporus*, Botulinolysin from *Clostridium botulinum*, Chauveolysin from *C. chauvoei*, Bifermentolysin from *C. bifermentans* , Sordellilysin from *C. sordellii* (see e.g. Palmer, 2001).

In some embodiments, the wild type cytolysin may be any cytolysin other than pneumolysin.

Preferred embodiments of all aspects of the invention include mutants of perfringolysin, intermedilysin or anthrolysin. They (or mutants of the other cytolysins described here) may comprise a mutation (e.g. a substitution or deletion) at a position corresponding to amino acid A146 of wild type PLY.

Preferred embodiments of all aspects of the invention include mutant perfringolysin, intermedilysin or anthrolysin comprising deletions or substitutions at positions equivalent to A146 and R147 of wild type pneumolysin, i.e. Δ6-PFO and Δ6-ILY.

In all aspects of the invention, where the mutant cytolysin is a mutant perfringolysin comprising a substitution or deletion at Y_{181}, and in particular, a Y_{181} to A_{181} substitution, it may comprise a further mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, wherein the further mutation is capable in isolation of reducing the toxicity of the wild type sequence. Thus the toxicity of the mutant will be lower than that of a perfringolysin mutant having only the mutation at position 181 of wild type perfringolysin.

Brief Description of the Figures

Figure 1 shows the amino acid sequences of wild-type cytolysin polypeptides. A. Pneumolysin from *Streptococcus pneumoniae;* B. Perfringolysin O from *Clostridium perfringens;* C. Intermedilysin from *Streptococcus intermedius;* D. Alveolysin from *Bacillus alvei;* E. Anthrolysin from *Bacillus anthracis;* F. Putative Cereolysin from *Bacillus cereus;* G. Ivanolysin O from *Listeria ivanovii;* H. Pyolysin from *Arcanobacterium pyogenes;* I. Seeligeriolysin O from *Listeria seeligeri;* J. Streptolysin O from *S. pyogenes;* K. Suilysin from *Streptococcus suis;* L. Tetanolysin from *Clostridium tetani;* M. Listeriolysin O from *Listeria monocytogenes.* All accession numbers are derived from NCBI-GenBank Flat File Release 141.0, April 15 2004.

Figure 2 shows a western blot of PLY deletion mutants detected by mAb PLY4;

Figure 3 shows the results of a quantitative haemolytic assay comparing WT PLY to Δ6 PLY mutant;

Figure 4 shows the results of a cytotoxicity assay comparing WT PLY to Δ6 PLY mutant;

Figure 5 shows electron micrographs of WT PLY treated erythrocyte membranes;

Figure 6 shows IL-6 levels in lung tissue after treatment with WT PLY or Δ6 PLY;

Figure 7 shows IL-6 levels in lung lavage after treatment with WT PLY or Δ6 PLY;

Figure 8 shows total protein levels in bronchoalveolar lavage after treatment with WT PLY or Δ6 PLY;

Figure 9 shows anti-PLY antibody levels in response to immunization of mice with WT PLY or Δ6 PLY;

Figure 10 shows the degree of haemolysis in relation to toxin concentration in SRBC (sheep red blood cell) treated with WT PLY or the deletion mutant ΔA146 PLY.

Figure 11 is a Western blot showing that polyclonal α-PLY antibodies also recognise PFO.

Figure 12 shows the degree of haemolysis in relation to toxin concentration in SRBC (sheep red blood cell) treated with Δ6 PFO and wild type PFO.

Figure 13 shows the degree of haemolysis in relation to toxin concentration in SRBC (sheep red blood cell) treated with Δ6 ILY and wild type ILY.

Figure 14 shows the degree of haemolysis in relation to toxin concentration in human erythrocytes treated with Δ6 ALO and wild type ALO;

Figure 15 compares the haemolytic activity of WT PLY and the mutants PLY W433F, Δ6 PLY, Δ7 PLY, Δ8 PLY and ΔA146 PLY;

Figure 16 shows the cytotoxicity to murine L929 fibroblasts of WT PLY and the mutants PLY W433F, Δ6 PLY, Δ7 PLY, Δ8 PLY and ΔA146 PLY;

Figure 17 shows that ΔA146 PLY does not cause degranulation of RBL-2H3 mast cells, while WT PLY does;

Figure 18 shows analysis of core body temperature following treatment with wild type PLY or ΔA146 PLY.

Detailed Description of the Invention

The amino acid sequences of a number of wild type cholesterol-binding cytolysins (CBCs) are shown in Figure 1. Figure 1 also indicates the GenBank identification number for each sequence. The invention is not restricted to mutants of

the cytolysins shown in Figure 1, but encompasses mutants of any cholesterol-binding cytolysin. A cholesterol-binding cytolysin is a molecule which, in the wild type state, is capable of binding to membrane cholesterol molecules, and has the ability to oligomerise and form pores in cholesterol-containing membranes. The wild type cytolysin may contain an amino acid sequence which has at least 33% amino acid identity, and preferably 40%, 50%, 60%, 70%, 80%, 90% or more amino acid identity to the region corresponding to amino acids 144 to 161 of the wild type pneumolysin protein, which has the consensus sequence VPARMQYEKITAHSMEQL (see Figure 1). Additionally or alternatively, the corresponding region of the cytolysin may be immunologically cross-reactive with amino acids 144 to 161 of the wild type pneumolysin protein. That is to say, antibodies reactive with the pneumolysin sequence will also bind to the corresponding cytolysin sequence, and *vice versa*.

The present invention relies on the identification of a number of cytolysin forms having a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin protein which have reduced toxicity as compared to the wild type cytolysin sequence, as reflected by a reduction in haemolytic activity and/or oligomerisation.

In one aspect, the present invention relates to mutant cytolysin proteins which differ from the wild type protein by the mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence. The consensus sequence of this region is as follows: VPARMQYE (see Figure 1).

The corresponding sequences for the cytolysins shown in Figure 1 are listed below.

Cholesterol Binding Cytolysin	**Consensus sequence to PLY (a.a. 144 - 161)**	**Amino acid position**
Pneumolysin	VPARMQYEKITAHSMEQL	V144 - L161

Perfringolysin O	LPARTQYSESMVYSKSQI	L175 - I192
Seeligeriolysin	INAKIDYSDEMAYSESQL	I201 - L218
Septicolysin	LPARTQYSESMVYSKSQI	L173 - I190
Streptolysin O	LPARTQYTESMVYSKSQI	L249 - I266
Intermedilysin	VPARMQYESISAQSMSQL	V202 - L219
Alveolysin	LPARLQYAESMVYSQNQI	L177 - I194
Anthrolysin	LPARTQYSESMVYSKSQL	L188 - L205
Cereolysin	LPARTQYSESMVYSKSQI	L175 - I192
Ivanolysin	ISAKIDYDQEMAYSESQL	I199 - L216
Suilysin	TQAELQYDETMAYSMSQL	T172 - L189
Tetanolysin	IPTRMSYSDTMVYSQSQL	I201 - L218
Listeriolysin O	VSAKIDYDDEMAYSESQL	V200 - L217

The mutant may have a substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 161, e.g. 144 to 151.

Thus, in all aspects of the invention, the mutant cytolysin may have a mutation, e.g. a substitution or deletion, at one or more of the amino acid residues corresponding to amino acids 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160 or 161 of wild type pneumolysin.

The invention further relates to mutant cytolysin proteins which differ from the wild type protein by the substitution or deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence. Examples of such double mutants are those which contain substitutions or deletions of amino acids corresponding to valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151, i.e. the corresponding amino acids shown in the table above.

These mutant cytolysin proteins are used *per se* in immunogenic compositions, together with one or more of a physiologically acceptable adjuvant, diluent or carrier.

Alternatively, these mutant cytolysin proteins are conjugated to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein, from the same or a heterologous organism as the cytolysin, to form conjugates

which are used in immunogenic compositions, together with one or more of a physiologically acceptable adjuvant, diluent or carrier. Thus the saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein to which the mutant protein is conjugated may be from *Streptococcus*, e.g. *Streptococcus pneumoniae*, *Streptococcus intermedius*, *Streptococcus suis*, *S. pyogenes*, *S. equisimilis*, *S. canis*, *Clostridium*, e.g. *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*, *Clostridium tetani*, *Clsotridium botulinum*, *Clostridium chauvoei*, *Clostridium bifermentans*, *Clostridium sordelli*, *Bacillus*, e.g. *Bacillus alvei*, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus laterosporus*, *Listeria*, e.g. *Listeria ivanovii*, *Listeria seeligeri*, *Listeria monocytogenes*, or *Arcanobacterium* e.g. *Arcanobacterium pyogenes* (see e.g. Palmer, 2001). The saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein may be derived from the bacterial capsule.

In either unconjugated or conjugated form, the mutant cytolysin proteins contained in immunogenic compositions are used in prophylaxis or therapy.

In either unconjugated or conjugated form, the mutant cytolysin proteins may further contain at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence. These further substitutions or deletions are described in Paton et al. published International Patent Application WO 90/06951.

According to a further aspect of the present invention, there is provided an immunogenic composition comprising an isolated mutant cytolysin protein as described herein.

The mutant cytolysin proteins may retain immunogenic activity in mammals. By "immunogenic in mammals" is meant that mammalian immune systems will produce antibodies to the mutant protein, and that these antibodies will also recognise wild type protein. Similarly, mammalian antibodies to the wild

type protein will also recognise the mutant protein. Preferably the mutant protein is immunogenic in humans. Preferably the mutant protein will stimulate the mammalian immune system to produce antibodies which bind to the wild type sequence corresponding to VPARMQYEKITAHSMEQL or VPARMQYE.

In one embodiment, the mutation is in the region of the cytolysin protein involved in oligomerisation of the wild type protein.

Without being bound by theory, it is believed that the mutant protein has reduced toxicity as a result of reduced pore-formation activity compared with wild type protein. This is believed to be associated with reduced oligomerisation activity and/or reduced haemolytic activity. Toxicity may be measured directly. Alternatively, one or more of pore formation, oligomerisation and haemolysis may be measured to provide an indication of likely toxicity.

Deletions and substitutions are examples of mutations which may be used to provide the mutant proteins of the invention with reduced toxicity. Non-conservative substitutions may be particularly suitable for reducing toxicity of the mutant, as a mutant having a non-conservative mutation is less likely to retain wild-type levels of function than one having a conservative substitution.

A conservative substitution may be defined as a substitution within an amino acid class and/or a substitution that scores positive in the BLOSUM62 matrix as shown below, thus a non-conservative substitution maybe defined as a substitution between amino acid classes, or which does not score positive in the BLOSUM62 matrix.

According to one classification, the amino acid classes are acidic, basic, uncharged polar and nonpolar, wherein acidic amino acids are Asp and Glu; basic amino acids are Arg, Lys and His; uncharged polar amino acids are Asn, Gln, Ser, Thr and Tyr; and non-polar amino acids are Ala, Gly, Val, Leu, Ile, Pro, Phe, Met, Trp and Cys.

According to another classification, the amino acid classes are small hydrophilic, acid/acidamide/hydrophilic, basic, small hydrophobic and aromatic, wherein small hydrophilic amino acids are Ser, Thr, Pro, Ala and Gly; acid/acidamide/hydrophilic amino acids are Asn, Asp, Glu and Gln; basic amino acids are His, Arg and Lys; small hydrophobic amino acids are Met, Ile, Leu and Val; and aromatic amino acids are Phe, Tyr and Trp.

Conservative substitutions, which score positive in the BLOSUM62 matrix, are as follows:

Original Residue	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W
Substitution	-	T A N	S	-	S	-	S D H	N E	D Q K	E R K	N Y	Q K	E Q R	I L V	M L V	M I V	M I L	Y W	H F W	F Y

Amino acid insertions within the region of amino acids 144 to 161, e.g. 144 to 151, may also be used to reduce toxicity of the PLY mutant. For example, insertions of 1, 2, 3, 4, 5, 10, 15, 20 or more amino acids may be used. However, deletions and substitutions are generally preferred to insertions as they are less likely to disrupt the wild type epitope; such disruption could reduce the immunogenicity of the mutant protein, which may be undesirable in an immunogenic composition.

The mutant cytolysin protein of the invention preferably has at least 80% amino acid identity with the corresponding wild type sequence, e.g. as shown in Figure 1. The mutant may have at least 85% identity, at least 90% identity, or at least 95% identity with the wild type sequence.

Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after

aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. % identity values may be determined by WU-BLAST-2 (Altschul et al., Methods in Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

Percent (%) amino acid similarity is defined in the same way as identity, with the exception that residues scoring a positive value in the BLOSUM62 matrix are counted. Thus, residues which are non-identical but which have similar properties (e.g. as a result of conservative substitutions) are also counted.

References in this specification to an amino acid of a first sequence "corresponding to" an amino acid of a second sequence should be construed accordingly. That is to say, residues which align with one another when the two sequences are aligned as described above, can be considered to correspond to one another.

In another aspect of the present invention, the mutant protein is conjugated to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein to form an immunogenic conjugate. In this aspect, the mutant cytolysin protein may retain its immunogenicity, or that immunogenicity may be ablated. In either event, the mutant cytolysin protein serves to enhance the immunogenicity of the saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein in the conjugate.

Such saccharides, oligosaccharides, polysaccharides, peptides, polypeptides or proteins are each conjugated to the mutant protein in any suitable manner, including, but not limited to: (1) direct coupling *via* protein functional groups (*e.g.*, thiol-thiol linkage, amine-carboxyl linkage, amine-aldehyde linkage; enzyme direct coupling); (2) homobifunctional coupling of amines (*e.g.*, using bis-aldehydes); (3) homobifunctional coupling of thiols (*e.g.*, using bis-maleimides); (4) homobifunctional coupling *via* photoactivated reagents (5) heterobifunctional coupling of amines to thiols (*e.g.*, using maleimides); (6) heterobifunctional coupling *via* photoactivated reagents (*e.g.*, the β-carbonyldiazo family); (7) introducing amine-reactive groups into a poly- or oligosaccharide *via* cyanogen bromide activation or carboxymethylation; (8) introducing thiol-reactive groups into a poly- or oligosaccharide *via* a heterobifunctional compound such as maleimido-hydrazide; (9) protein-lipid conjugation *via* introducing a hydrophobic group into the protein and (10) protein-lipid conjugation *via* incorporating a reactive group into the lipid. Also, contemplated are heterobifunctional "non-covalent coupling" techniques such the Biotin-Avidin interaction. For a comprehensive review of conjugation techniques, *see* Aslam and Dent (1998), incorporated hereinafter by reference in its entirety.

Further methods of conjugating a peptide, polypeptide or protein to a protein are described in U.S. provisional patent applications 60/530,480 and 60/530,481, both filed December 17, 2003, and both incorporated by reference in their entirety.

Additionally, US Patent 5,565,204 to Kuo et al. described a method for conjugating such polysaccharides to the wild type PLY protein; that method is also suitable for conjugating such polysaccharides to the mutant cytolysin proteins of this invention.

The immunogenic compositions of the present invention may be conjugated immunogenic compositions. Each immunogenic composition may comprise one or more saccharides, oligosaccharides, polysaccharides, peptides, polypeptides or proteins, which may be derived from the source organism of the wild type cytolysin protein. In non-limiting examples, such components may be derived from the capsule of the organism.

In one embodiment, the saccharides, oligosaccharides or polysaccharides are derived from more than one serotype of the source organism; the particular serotypes will depend on the intended use for the immunogenic composition and the prevalence of these serotypes in the target population.

Alternatively, the saccharides, oligosaccharides, polysaccharides, peptides, polypeptides or proteins are derived from a heterologous organism (that is, an organism other than that from which the cytolysin molecule is derived). In the case of saccharides, oligosaccharides or polysaccharides, multiple serotypes may be obtained from, without limitation, *Neisseria meningitidis* (for example, from serotypes A, C, Y and W135), *Staphylococcus aureus* and *Haemophilus influenzae*.

In certain aspects of the invention, the mutant cytolysin protein is conjugated to another peptide, polypeptide or protein of the same species or strain of organism. Alternatively, the mutant cytolysin protein is conjugated to a peptide, polypeptide or protein from a heterologous organism, including a human. For example, the mutant protein is conjugated to another peptide, polypeptide or protein, which is from a pathogenic virus, bacterium, fungus or parasite, or (2) from a cancer cell or tumor cell, or (3) from an allergen so as to interfere with the production of IgE so as to moderate allergic responses to the allergen, or (4) from amyloid precursor protein (APP) so as to prevent or treat disease characterized by amyloid deposition in a vertebrate host.

The moiety of APP which is conjugated to the mutant protein may be the β-amyloid peptide (also referred to as Aβ peptide), which is an internal, 39-43 amino acid fragment of (APP), which is generated by processing of APP by the β and γ secretase enzymes. An example of such a peptide is the Aβ1-42 peptide, which has the following amino acid sequence:
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala

The Aβ component may be further administered in the form of a fragment conjugated to the mutant protein. Non-limiting examples of such fragments include Aβ1-3, 1-4, 1-5, 1-6, 1-7, 3-7, 3-8, 3-9, 3-10, 3-11, 1-10 and 1-12. The use of Aβ and fragments thereof, as conjugated to proteins other than PLY, is described in published International Patent Applications WO 99/27944 and WO 00/72880, which are hereby incorporated by reference.

A further aspect of the present invention provides a method of prophylaxis or treatment for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition comprising an isolated cytolysin protein having a mutation as described herein, where the mutant cytolysin protein is unconjugated or conjugated as described herein. Typically the method is intended for prophylaxis or treatment of infection by one or more species or strains of bacteria having a cholesterol-binding cytolysin which is immunologically cross-reactive with the relevant wild-type cytolysin.

The mode of administration of an immunogenic composition of the invention, whether of the mutant cytolysin protein alone or as part of an immunogenic conjugate, may be by any suitable route which delivers an immunoprotective amount of the protein to the subject. One such route is the parenteral route, such as by intramuscular or subcutaneous administration. Other modes of administration may also be

employed, where desired, such as the mucosal route, such as by oral, rectal, buccal or intranasal administration, or via other parenteral routes, i.e., intradermally or intravenously.

Generally, the immunogenic composition will usually be presented as a pharmaceutical formulation including a physiologically acceptable carrier or excipient, for example, sterile water or sterile isotonic saline, as well as any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with administration to humans. The appropriate carrier will be evident to those skilled in the art and will depend in large part upon the route of administration. The immunogenic composition of the present invention may also include a physiologically acceptable diluent such as sterile water or sterile isotonic saline. The formulation may be prepared by conventional means.

It will be understood, however, that the specific dose level for any particular recipient mammal will depend upon a variety of factors including age, general health, and sex; the time of administration; the route of administration; synergistic effects with any other drugs being administered; and the degree of protection being sought. Of course, the administration can be repeated at suitable intervals if necessary.

The mammal may be a human, or may be a non-human mammal. The immunogenic composition may be administered in any convenient manner; for example, those described above.

The immunogenic composition of the present invention may include one or more physiologically acceptable adjuvants. A substance that enhances the immune response when administered together with an immunogen or antigen is known as an adjuvant. A number of cytokines or lymphokines have been shown to have immune modulating activity, and thus may be used as adjuvants, including, but not limited to, the interleukins 1-α, 1-β, 2, 4, 5, 6, 7, 8, 10, 12 (see, e.g., U.S. Patent No. 5,723,127, which is hereby incorporated by reference), 13, 14, 15, 16, 17

and 18 (and its mutant forms), the interferons-α, β and γ, granulocyte-macrophage colony stimulating factor (GM-CSF; see, e.g., U.S. Patent No. 5,078,996, which is hereby incorporated by reference, and ATCC Accession Number 39900), macrophage colony stimulating factor(M-CSF), granulocyte colony stimulating factor (G-CSF), and the tumor necrosis factors α and β. Still other adjuvants useful in this invention include a chemokine, including without limitation, MCP-1, MIP-1α, MIP-1β, and RANTES. Adhesion molecules, such as a selectin, e.g., L-selectin, P-selectin and E-selectin may also be useful as adjuvants. Still other useful adjuvants include, without limitation, a mucin-like molecule, e.g., CD34, GlyCAM-1 and MadCAM-1, a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95, a member of the immunoglobulin superfamily such as PECAM, ICAMs, e.g., ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3, co-stimulatory molecules such as CD40 and CD40L, growth factors including vascular growth factor, nerve growth factor, fibroblast growth factor, epidermal growth factor, B7.2, PDGF, BL-1, and vascular endothelial growth factor, receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and DR6. Still another adjuvant molecule includes Caspase (ICE). See, also International Patent Publication Nos. WO98/17799 and WO99/43839, incorporated herein by reference.

Suitable adjuvants used to enhance an immune response further include, without limitation, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, MT), which is described in U.S. Patent No. 4,912,094, which is hereby incorporated by reference. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, MT), and which are described in United States Patent No. 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino] ethyl 2-Deoxy-4-O-

phosphono-3-O-[(R)-3-tetradecanoyoxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoyl-amino]-β-D-glucopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form or as a stable emulsion.

Still other adjuvants include mineral oil and water emulsions, aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic polyols, muramyl dipeptide, killed *Bordetella*, saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA.), described in U.S. Patent No. 5,057,540, which is hereby incorporated by reference, and particles generated therefrom such as ISCOMS (immunostimulating complexes), *Mycobacterium tuberculosis*, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Patent No. 6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, PT-K9/G129; see, e.g., International Patent Publication Nos. WO 93/13302 and WO 92/19265, incorporated herein by reference.

Also useful as adjuvants are cholera toxins and mutants thereof, including those described in published International Patent Application number WO 00/18434 (wherein the glutamic acid at amino acid position 29 is replaced by another amino acid (other than aspartic acid), preferably a histidine). Similar CT toxins or mutants are described in published International Patent Application number WO 02/098368 (wherein the isoleucine at amino acid position 16 is replaced by another amino acid, either alone or in combination with the replacement of the serine at amino acid position 68 by another amino acid; and/or wherein the valine at amino acid position 72 is replaced by another amino acid). Other CT toxins are described in published International Patent Application number WO 02/098369 (wherein the arginine at amino acid position 25 is replaced by another amino acid; and/or an amino acid is

inserted at amino acid position 49; and/or two amino acids are inserted at amino acid positions 35 and 36).

The haemolytic activity of the mutant cytolysin protein may be determined in any suitable manner. One particular protocol as used in the present invention is as follows. Toxin was prepared in serial dilutions in 1.5ml 1 x PBS (Oxoid). An equal volume of 2% (vol/vol) SRBC (sheep red blood cell) or HRBC (human red blood cell) was added to each dilution and incubated at 37°C for 30 minutes. Solutions were then centrifuged at 3000rpm for 5 minutes to pellet SRBC membranes or whole cells. Haemoglobin content of the supernatant was read at OD540nm and plotted against toxin concentration to give the degree of haemolysis in relation to toxin concentration. An OD540nm of 0.5 = 50% lysis.

Preferably the mutant cytolysin protein is non-haemolytic at concentrations of more than 1 µg/ml; more preferably at concentrations of more than 5 µg/ml; still more preferably at more than 10 µg/ml, at more than 25 µg/ml, or at more than 35 µg/ml; and most preferably at more than 50 µg/ml. Determination of haemolysis may be carried out as described above.

Determination of pore-forming activity may be determined in any suitable manner; a preferred protocol relies on visual inspection of SRBC or HRBC membranes by means of electron microscopy; this allows the number of pores to be visualised. This protocol is described in more detail below.

Several methods may be employed to analyse oligomerising activity of pore-forming toxins. For example, analytical ultracentrifugation, as described by Morgan et al (1993), can be used to study oligomerisation of toxins in solution. A sucrose density gradient can be applied to toxin bound erythrocytes in which oligomers are observed in the high molecular weight fractions and separated from other erythrocyte membrane proteins (Bhakdi et al, 1985; Saunders et al, 1989).

One particular method of comparing the oligomerisation activity of mutant cytolysin in solution with that of wild type protein is to use a fluorescence assay conducted in a similar manner to that described by Search (2002). Briefly, ANS (8-anilino-1-napthalene-sulphonic acid) (Kodak Ltd.) binds as an extrinsic fluor to the toxin. In aqueous solution, ANS has weak fluorescence at 490nm (read with JASCO FP-750 spectrofluorometer) but in a hydrophobic environment ANS fluorescence increases. This phenomenon allows the movement of ANS bound monomers to be tracked in solution. Sodium deoxycholate (BDH Laboratory supplies) can be used to induce oligomerisation. An increase in fluorescence is observed when wild type toxin plus ANS is treated with sodium deoxycholate as the toxin self-associates bringing the ANS from a hydrophilic to hydrophobic environment. Derivatised toxin, that is toxin chemically modified with dithio(bis)nitrobenzoate to remain monomeric, does not result in an increase in fluorescence at 490nm when treated with sodium deoxycholate. From this experiment, it is predicted that mutant cytolysin would give the same result as derivatised cytolysin if it remains monomeric. If mutant cytolysin is found to fluoresce to the same extent as wild type, then it can be concluded that the mutant does oligomerise.

The toxicity of the proteins and compositions of the invention may be determined directly, by administering the mutant to a non-human test mammal, e.g. a rodent. The toxicity of the mutant may be compared with that of the wild type protein. Suitable indicators of toxicity include survival, animal behaviour, and inflammation (which may be determined by measuring inflammatory cytokine production, e.g. in bronchoalveolar lavage). Suitable protocols are described below in the Examples.

The present invention further provides a method of preparation of an immunogenic composition, the method comprising the steps of:

providing an isolated mutant cytolysin protein with the mutations described herein and having reduced haemolytic activity compared with wild type cytolysin protein, the mutant protein being antigenic in mammals; and

conjugating the mutant protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein.

According to a further aspect of the present invention, there is provided an isolated and purified nucleic acid sequence comprising an isolated nucleic acid sequence encoding a mutant cytolysin protein wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein, or which is complementary to such a nucleic acid sequence, the mutant protein being immunogenic in mammals. Further aspects of the invention provide nucleic acid sequences which are complementary to such sequences.

Nucleic acid sequences can be derived from protein sequences based on the degeneracy of the genetic code.

Nucleic acid sequences of the present invention may comprise additional regulatory sequences, for example, promoters or repressors. The nucleic acid sequences may be comprised in an expression vector, for example, plasmids, artificial chromosomes, expression cassettes and the like.

According to a still further aspect of the present invention, there is provided a recombinant host cell transformed, transfected or infected with a recombinant expression vector comprising an isolated and purified nucleic acid sequence expressing a mutant cytolysin protein as described herein. In one embodiment, the cell is a prokaryotic cell.

According to a still further aspect of the present invention, there is provided a method of screening candidate

mutant cytolysin proteins for suitability for use in immunogenic compositions, the method comprising the steps of:

providing a mutant cytolysin protein;

testing the mutant protein for haemolytic activity;

testing the mutant protein for oligomerisation activity; and

comparing the haemolytic and oligomerisation activity of the mutant protein with those of a non-mutant (e.g. a wild type) protein.

Those mutant proteins which have reduced haemolytic and oligomerisation activity compared with the non-mutant form of the protein will be likely to be good candidates for the preparation of immunogenic compositions.

The method may further comprise the step of testing the mutant protein for immunogenic activity in a target mammal. This may comprise the step of contacting the mutant protein with an antibody to the non-mutant protein. This may be performed *in vivo* or *in vitro*.

The invention described herein relates to deletion mutants of cytolysins which exhibit reduced toxicity, haemolysis and pore formation. The data demonstrate that at 7µg/dose, Δ6 PLY is not detrimental to mice compared to 2µg/dose of WT PLY.

Nevertheless, various mutations created by the present inventors surrounding the PLY N_{143} residue and described herein are still recognised by Western blotting with mAb PLY 4, indicating that this highly antigenic site on PLY has not been altered. The site of mutation has been shown to be highly antigenic by epitope scanning and is recognised by both human sera and rabbit hyper-immune sera (Salo et al, 1993).

All PLY mutants created were confirmed to be forms of pneumolysin. The fact that mAb PLY4 recognises the mutants indicates that the epitope has not been altered to such an extent that it is no longer specific for this antibody. Larger deletions within this area should create mutants that are not recognised by mAbPLY4. As this region has been identified as

being highly immunogenic (Salo et al, 1993) it is useful that the site remains intact in the deletions that we have created in terms of use in immunogenic compositions.

The non-toxic mutants described herein are within the site proposed to be involved in oligomerisation (de los Toyos et al, 1996). Further *in vitro* characterisation of one purified toxoid, Δ6 PLY, revealed that it was not cytotoxic to murine fibroblasts or erythrocytes. This suggests that host cell membranes are not lysed by Δ6 PLY because oligomerisation (pore formation) has been prevented. Pores on SRBC membranes treated with WT PLY are readily visible, but pores have not been observed on membranes treated with Δ6 PLY. There were difficulties in fixing Δ6 PLY treated membranes to the grids for visualisation with the EM. This may be due to the agglutination of membranes that are seen in haemolytic assays with Δ6 PLY. The haemagglutination effect observed in haemolytic assays of Δ6 PLY with SRBC suggests that Δ6 PLY monomers still bind to host cell membranes. A labelled form of Δ6 PLY was created which allows visualisation of binding to host cell membranes. From binding assays (data not shown) it was confirmed that Δ6 PLY did bind to the host cell membrane. There may be a weak affinity between the Δ6 PLY monomers, allowing cross-linking of monomers but not formation of true oligomers. This cross-linking of Δ6 PLY monomers in addition to monomers binding to erythrocytes could create the matrix observed in 96-well plates. It is proposed that by creating the Δ6 PLY mutant, the oligomerisation stage was blocked, but host cell binding/recognition was not abolished.

This hypothesis is supported by the finding that Δ146 PLY is still capable of associating with cell membranes as determined using eGFP-tagged versions of the mutant and wild type proteins, but that Δ146 PLY does not form pores in cell membranes. Instead, long chains of protein are seen at the cell surface, which may be self-associated protein which is unable to oligomerise correctly to form pores.

In vivo treatment with Δ6 PLY did not result in an increase of the inflammatory cytokine IL-6 24 hours post-treatment. Mice treated with WT PLY were found to produce 10 times more IL-6 than the saline control and Δ6 PLY treatment. The data established that treatment with Δ6 PLY did not induce the inflammatory side effects that are associated with native PLY. WT PLY treatment resulted in a localised inflammatory response at the site of administration. This localised IL-6 production in the bronchoalveolar lavage is likely to be from recruited neutrophils (Kadioglu, 2000) and alveolar macrophages that produce more IL-6 than the epithelial cells of the lung tissue (Kerr, personal communication 2003).

Wild type PLY severely damaged lung integrity, but lungs treated with Δ6 PLY remained healthy. The large amount of total protein observed in the airways of WT PLY treated mice has been characterised as an influx of host proteins (Rubins & Janoff, 1998). PLY has previously been implicated in the disruption of tight junctions (Rayner et al, 1995), allowing host proteins to 'leak' into the airways via the disruption of the capillary/airway barrier. A low inflammatory response and no disruption to the lungs by Δ6 PLY correlate with Δ6 PLYs inability to create pore-forming oligomers in host cell membranes.

Treatment of mice with WT PLY has also been demonstrated to cause a sustained hypothermic response which is not seen in animals treated with Δ146 PLY.

Mutations equivalent to Δ6 PLY have also been constructed in Perfringolysin O (PFO) and Intermedilysin (ILY). Both of these mutants were observed to have the same phenotype as Δ6 PLY. This suggests that the results obtained with PLY can be extrapolated to other cytolysins.

These and other aspects of the present invention will now be described by way of the following non-limiting examples, and with reference to the accompanying Figures.

Examples

Example 1

Site-directed Mutagenesis of Pneumolysin

Eight double amino acid deletions from wild type pneumolysin were created using the Quikchange® site directed mutagenesis kit (Stratagene). The template plasmid was the high expression vector pKK233-3 (Clontech Laboratories) in which PLY was previously inserted. Primers designed to delete the relevant amino acids (see Table 1 below) were ordered from Sigma-Genosys. The following deletions were created to span the N_{143} region of PLY:

$W_{134}H_{135}Q_{136}D_{137}Y_{138}G_{139}Q_{140}V_{141}N_{142}N_{143}V_{144}P_{145}A_{146}R_{147}M_{148}Q_{149}Y_{150}E_{151}$; where

(Δ1) $W_{134}H_{135}$, (Δ2) $Q_{136}D_{137}$, (Δ3) $Y_{138}G_{139}$, (Δ4) $Q_{140}V_{141}$, (Δ5) $V_{144}P_{145}$, (Δ6) $A_{146}R_{147}$, (Δ7) $M_{148}Q_{149}$, (Δ8) $Y_{150}E_{151}$ (see Table 2). $N_{142}N_{143}$ was the deletion previously created (Search (2000)), where proteinase K cuts PLY into two fragments.

Table 1 : Primers used to create double amino acid deletions within the PLY gene

Primers	Nucleotide sequences of primers for site-directed mutagenesis
Δ1 fwd	5′-CGATTTGTTGGCTAAGCAAGATTATGGTCAGG-3′
Δ1 rev	5′-CCTGACCATAATCTTGCTTAGCCAACAAATCG-3′
Δ2 fwd	5′-GTTGGCTAAGTGGCATTATGGTCAGGTCAATAATGTCCC-3′
Δ2 rev	5′-GGGACATTATTGACCTGACCATAATGCCACTTAGCCAAC-3′
Δ3 fwd	5′-GGCTAAGTGGCATCAAGATCAGGTCAATAATGTCCC-3′
Δ3 rev	5′-GGGACATTATTGACCTGATCTTGATGCCACTTAGCC-3′
Δ4 fwd	5′-GGCATCAAGATTATGGTAATAATGTCCCAGCTAG-3′
Δ4 rev	5′-CTAGCTGGGACATTATTACCATAATCTTGATGCC-3′
Δ5 fwd	5′-GGTCAGGTCAATAATGCTAGAATGCAGTATG-3′
Δ5 rev	5′-CATACTGCATTCTAGCATTATTGACCTGACC-3′
Δ6 fwd	5′-GGTCAATAATGTCCCAATGCAGTATGAAAAAATAACGGCTC-3′
Δ6 rev	5′-GAGCCGTTATTTTTTCATACTGCATTGGGACATTATTGACC-3′
Δ7 fwd	5′-GGTCAATAATGTCCCAGCTAGATATGAAAAAATAACGGCTC-3′
Δ7 rev	5′-GAGCCGTTATTTTTTCATATCTAGCTGGGACATTATTGACC-3′
Δ8 fwd	5′-GTCCCAGCTAGAATGCAGAAAATAACGGCTCACAGC-3′
Δ8 rev	5′-GCTGTGAGCCGTTATTTTCTGCATTCTAGCTGGGAC-3′

Note - Reverse primers [rev] are the exact complement and reverse of the forward [fwd] primers with the bases to be deleted removed from the primer.

Table 2 : Bases deleted for each mutation within the PLY gene and amino acids deleted.

Deletion	Bases deleted within the PLY gene	Amino acids deleted
Δ1	TGGCAT (bp400-405)	$W_{134}H_{135}$
Δ2	CAAGAT (bp406-411)	$Q_{136}D_{137}$
Δ3	TATGGT (bp412-417)	$Y_{138}G_{139}$
Δ4	GAGGTC (bp418-423)	$Q_{140}V_{141}$
Δ5	GTCCCA (bp430-435)	$V_{144}P_{145}$
Δ6	GCTAGA (bp436-441)	$A_{146}R_{147}$
Δ7	ATGCAG (bp442-447)	$M_{148}Q_{149}$

Δ8	TATGAA (bp448-453)	$Y_{150}E_{151}$

Example 2

Protein expression and purification

Wild type (WT) and mutant PLY was expressed in *Escherichia coli* and harvested as described previously (Mitchell et al, 1989). Cells were disrupted using the benchtop cell disrupter (Constant Systems Ltd) and cytoplasmic proteins obtained by centrifugation at 13,000 rpm for 30 minutes. Hydrophobic Interaction Chromatography with a phenyl ether matrix (PE20, Applied Biosystems) was used to purify PLY with the BioCAD (RTM) 700E Perfusion Chromatography Workstation (Applied Biosystems). Eluted fractions were run on SDS-PAGE and coomassie stained using standard protocol and fractions containing pure PLY were pooled.

Example 3

Quantitative Haemolytic Assay

Haemolytic activity of purified protein was assessed using an assay based on that reported by Walker et al., (1987) using a 2% (vol/vol) sheep red blood cell (SRBC) (E & O laboratories) or human red blood cell (HRBC) solution in 1 x Phosphate Buffered Saline (PBS) (Oxoid). Pooled fractions were concentrated using minicon B15 clinical sample concentrators (Millipore). Toxin was prepared in serial dilutions in 1.5ml 1 x PBS (Oxoid). An equal volume of 2% (vol/vol) SRBC was added to each dilution and incubated at 37°C for 30 minutes. Solutions were then centrifuged at 3000rpm for 5 minutes to pellet SRBC membranes or whole cells. Haemoglobin content of the supernatant was read at OD540nm and plotted against toxin concentration to give the degree of haemolysis in relation to toxin concentration. An OD540nm of 0.5 = 50% lysis.

Crude haemolytic analysis revealed that four of these mutants, deletions 5-8, were non-haemolytic. Further analysis of purified Δ6 PLY ($A_{146}R_{147}$) in a quantitative haemolytic assay

(Fig. 3) revealed that it was non-lytic at concentrations of 50µg/ml whereas <1µg/ml of WT PLY was haemolytic to SRBC. Purified preparations of Δ5 PLY and Δ6 PLY were observed to agglutinate erythrocytes in 96-well micro-titre plates but not lyse the cells; this effect was concentration dependent.

Example 4

Electron Microscopy

200µl 2% (v/v) SRBC solution was incubated with an equal volume of 0.2mg/ml WT PLY or Δ6 PLY at 37°C for 20 minutes then centrifuged with benchtop centrifuge to pellet the SRBC membranes. Membranes were washed with dH_2O x 3 and resuspended in 100µl dH_2O. 5µl of suspension was fixed onto carbon-coated grids and negatively stained with 1% phosphotungstate acid, pH6.8. Magnification was at x 25000 using an LEO 912 Energy Filter Transmission Electron Microscope.

30-40µm pores were visualised on erythrocyte membranes treated with 0.2mg/ml WT PLY (Fig. 5). In contrast, pores were not visualised on membranes treated with 0.2mg/ml Δ6 PLY.

Example 5

Western Blotting

PLY mutants created by site-directed mutagenesis were detected in Western blots using standard techniques. Blots were incubated with polyclonal anti-PLY serum from rabbit or monoclonal PLY 4 anti-PLY serum from mouse (de los Toyos et al, 1996) and then incubated with the relevant HRP-linked antibody (Amersham Life Sciences) and developed.

Of the eight double amino acid deletions created, all were recognised by Western blotting with polyclonal anti-PLY serum (not shown) and by mAb PLY 4 (Fig. 2) prepared by de los Toyos et al (1996).

Example 6

L929 Killing Assay

L929 murine fibroblasts (ECACC, no.85011425) were cultured in RPMI 1640 media + 10% Foetal Bovine Serum (FBS) (Gibco), passaged and transferred to a 96-well plate and incubated for 24h at 37°C, 5% CO_2. Serial dilutions of purified WT PLY and mutant Δ6 PLY toxin were prepared in RPMI 1640 media from a stock concentration of 0.05mg/ml and added to the L929 fibroblasts. Cell viability upon 24h incubation with PLY was assessed using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrzolium bromide) (Sigma) which is degraded by mitochondrial activity into a purple formazan precipitate. MTT in wells with dead cells will remain yellow. Optical density was read at 540nm with an MRX plate reader (Dynatech Laboratories).

Cytotoxicity assays with L929 murine fibroblasts were run to assess the toxicity of mutant Δ6 PLY compared to WT PLY (Fig. 4). At concentrations of 30µg/ml, Δ6 PLY was not toxic to fibroblasts, whereas < 500 pg/ml of WT PLY was cytotoxic.

Example 7

In vivo Cytokine Analysis

Eight week-old MF-1 mice (Harlan) were lightly anaesthetised with 2% halothane/1.5% oxygen (1.5 litre/min) (Zeneca). Purified LPS-free WT PLY was administered intranasally at 2µg/dose and Δ6 PLY at 7µg/dose (9.928ng LPS/dose) in 50µl volumes with a saline group as a control (n= 4 for each treatment). Lipopolysaccharide (LPS) content of purified toxin was determined using the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit (BioWhittaker) and run according to manufacturer's instructions. Mice were monitored to a 24h end-point. Serum, bronchoalveolar lavage and lung tissue samples were recovered and processed as described previously (Kerr, et al. 2002). Cytokine levels were measured with commercial cytokine ELISA kits for Interleukin (IL)-6, Interferon (IFN)-γ (Pharmigen) and Tumor Necrosis Factor

(TNF)-α (R&D systems, UK). Total protein levels in the lavage were measured using standard Bradford Assay.

Non-parametric analysis by Mann-Whitney U test was used to measure cytokine and total protein levels where $p < 0.05$ was considered statistically significant. Values are expressed as medians ± 1 median absolute deviation (MAD) using Statview (Abacus Concepts).

As part of *in vivo* toxicity studies, gross symptoms were ascertained. All mice survived 24 hours post-treatment except one from the WT PLY group. Mice treated with Δ6 PLY and saline recovered from the anaesthetic quicker than mice given WT PLY. Behaviour of Δ6 PLY treated mice was similar to that of the saline control but WT PLY treated mice exhibited piloerection, laboured breathing and a hunched stance over a 6- hour period, recovering within the 24-hour time scale.

Next, an inflammatory cytokine analysis was performed. IL-6 production was measured as a marker of toxicity of PLY to the host. There was a greater than 10-fold increase in IL-6 levels in the bronchoalveolar lavage of WT PLY treated mice (Fig. 7) compared to Δ6 PLY treatment ($p<0.05$) and the saline control ($p<0.05$). Treatment with WT PLY induces inflammation in the host airways whereas treatment with Δ6 PLY does not. The median IL-6 level in WT treated bronchoalveolar lavage was 416pg/ml (range of 335-2225pg/ml) whereas the background IL-6 level was low (59pg/ml) with no increase in mice treated with Δ6 PLY (36pg/ml) (see Table 3 below). An increase in IL-6 levels was observed in lung tissue of WT treated mice ($p<0.05$) compared to the saline control (Fig. 6). There was no significant IL-6 increase in lung tissue of Δ6 treated mice compared to the saline treatment. Measurements of IFN-γ and TNF-α were not significant between treatments 24h post-administration (data not shown).

Table 3: IL-6 median (min-max) levels in bronchoalveolar lavage 24h post treatment

Treatment (i.n.)	Lung Tissue (pg/ml)	Lung Lavage (pg/ml)	P values (*=significant)		
				Lung	Lavage
NaCl	117 (103-139)	59 (19-113)	NaCl/Δ6	0.3865	0.772
Δ6	147 (73-209)	36 (30-132)	Δ6/WT	0.1573	0.033
Wild type (WT)	171 (168-448)	416 (335-2225)	NaCl/WT	0.0339*	0.033

Total protein levels (Fig. 8) were measured in the bronchoalveolar lavage to assess lung integrity. Increases in protein levels were not observed for Δ6 PLY treated mice compared to healthy lavage samples. Airways of WT PLY treated mice had large amounts of protein (3.57mg/ml) in them compared to a background total protein level of 0.23mg/ml for the saline control group (Fig. 8).

Example 8

Mouse Immunogenicity Studies

A mouse immunogenicity study was performed to compare responses of the wild type PLY protein to the Δ 5, 6 and 7 mutant proteins. All immunogenic compositions were prepared at 5 μg rPLY/dose in the presence of a combination of adjuvants, $AlPO_4$ (0.2 mg) and MPL-SE (50 μg). $AlPO_4$ (0.2 mg) and MPL-SE (50 μg) in phosphate-buffered saline (PBS) was used as a negative control.

Groups of 5 female, CD-1 mice, age 6-8 weeks, were immunized intraperitoneally and received 2 booster doses at 2 week intervals. Blood was collected retro-orbitally at weeks 0, 2, 4, and 6. Individual serum was assayed and GMTs represented an end point at 0.3. Week 0 antibodies were all <50. As shown in Table 4, the three PLY mutants elicited antibodies in the mice which were comparable to those elicited by wild type PLY.

Table 4: Serum IgG Antibody Responses to Mutant Recombinant Pneumolysin (rPLY) in Mice

Immunogen	rPL Dose (µg)	GMT Antibodies for rPL Week		
		2	4	6
PBS	-	50	50	50
rPL (nlrPL 01-01)	5.0	16,456	806,776	3,104,055
rPL Wild type (PLY C73)	5.0	11,420	578,645	997,727
rPL Δ5	5.0	3,892	424,253	1,375,535
rPL Δ6	5.0	18,679	999,574	1,452,972
rPL Δ7	5.0	15,920	2,266,935	1,246,988

Example 9

Generation of anti-PLY Antibodies

Levels of anti-PLY antibodies raised in mice immunized with wild type PLY or Δ6 PLY were determined by immunizing MF-1 mice with an initial subcutaneous injection with 20µg WT PLY or Δ6 PLY, each with 100µg Alum/ 100µl dose. Mice were then boosted twice with the same dosage. Serum was collected on day 47 of the immunization protocol and analysed for anti-PLY IgG antibody. Antibody dilution curves are displayed in Figure 9 as the group mean OD_{490}nm ± SEM against the serum serial dilution. An initial dilution of serum to 1/1000 was used as more concentrated samples resulted in complete saturation of the substrate. Figure 9 demonstrates that high levels of antibodies were produced in response to both Δ6 PLY + Alum and WT PLY+ Alum, but not to the Alum only control group.

Next, the ability of anti-PLY antibodies to neutralise the haemolytic activity was assessed. Anti-PLY antibodies in the Δ6 PLY and wild type PLY treated groups were observed to

completely neutralise 2.5 Haemolytic Units (HU) of PLY to a titre of 1000-2400 in a haemolytic assay (where neutralising ability is expressed as the reciprocal of the antibody dilution that completely neutralises 2.5HU of PLY) (data not shown). This demonstrates that a neutralising site on PLY is recognised and bound to by the antibodies produced in response to immunisation with Δ6 PLY + Alum and WT PLY + Alum. Because Δ6 PLY is non-toxic and does not induce the *in vivo* levels of cytokine production observed with wild type PLY treatment, Δ6 PLY is therefore a more favourable protein than wild type PLY for use as an immunogenic carrier protein.

Example 10

Effect of a Single Amino Acid Deletion

A mutant PLY was generated which had a single amino acid deletion: the alanine at amino acid 146 was deleted (ΔA146 PLY). As shown in Figure 10, this single deletion (ΔA146) also resulted in a non-haemolytic form of PLY. ΔA146 PLY was not haemolytic to SRBC to concentrations >100μg/ml, whereas wild type PLY was haemolytic at concentrations <1μg/ml. Production of this mutant was confirmed by sequencing and by Western blotting of the expressed protein with polyclonal anti-PLY serum (data not shown).

Example 11

Mutation of other cytolysin polypeptides

Mutants of perfringolysin O (PFO) and intermedilysin (ILY) were constructed, each having a deletion equivalent to Δ6 of PLY. Thus in Δ6 PFO, alanine at position 177 and arginine at position 178 are deleted (see Fig. 1B); in Δ6 ILY, alanine at position 204 and arginine at position 205 are deleted (see Fig. 1C).

Table 5: Primers used to create double amino acid deletions within the PFO and ILY genes.

Primers	Nucleotide sequences of primers for site-directed mutagenesis
Δ6 PFO fwd:	5′- CTACACATACTTTACCAACTCAATATTCAGAATCTATGG
Δ6 PFO rev:	5′- CCATAGATTCTGAATATTGAGTTGGTAAAGTATGTGTAG
Δ6 ILY fwd:	5′- CTAAAACTCATGCTGTACCAATGCAATATGAATCTATTAGC
Δ6 ILY rev:	5′- GCTAATAGATTCATATTGCATTGGTACAGCATGAGTTTTAG

Native and Δ6 perfringolysin (PFO) are both detected by polyclonal α-PLY antibodies (Fig. 11) highlighting the similarity between PLY and PFO. Such data suggest that antibodies produced in response to immunisation with one Cholesterol Dependent Cytolysin (e.g. PLY) may confer cross-protection against another CDC.

Δ6 PFO and Δ6 ILY are both are non-haemolytic in comparison to their wild type derivatives (Fig. 12 and Fig. 13).

Example 12

Mutation of mature Anthrolysin O

The 34 amino acid signal sequence of anthrolysin O from *B. anthracis* was removed to give the mature protein (mALO), starting with the sequence ETQ.

A mutant of the mature anthrolysin O polypeptide was then constructed having a deletion equivalent to Δ6 of PLY. Thus in Δ6 mALO, alanine 156 and arginine 157 (corresponding to

A190 and R191 in full-length wild type ALO) are deleted (see Fig. 1C).

Table 6: Primers used to create ΔmALO.

Primers	Nucleotide sequences of primers for site-directed mutagenesis
Δ6 mALO fwd:	5'- CAACACATACGTTACCTATGCAGTATACAGAATC
Δ6 mALO rev:	5'- GATTCTGTATACTGCATAGGTAACGTATGTGTTG

Δ6 mALO was found to be non-haemolytic against human erythrocytes as compared to the mature wild-type protein (Figure 14).

Example 13

Haemolytic activity of PLY mutants in comparison to PLY W433F

The haemolytic activity of the deletion mutants Δ6, Δ7, Δ8 PLY and ΔA146 PLY against human erythrocytes was compared with that of WT PLY and PLY mutant carrying the substitution W433F, which has previously been described to possess only 1% of the haemolytic activity of WT PLY (see WO90/06951).

Figure 15 shows that, as expected, the W433F mutant shows ~1% of the haemolytic activity of wild type PLY. However the deletion mutants do not cause lysis of human erythrocytes at all.

Example 14

Binding of GFP-tagged PLY to erythrocyte membranes

Fluorescence microscopy was used to visualise erythrocytes treated with eGFP-tagged forms of WT PLY and Δ6 PLY.

Erythrocyte ghosts were prepared from human blood by repeated washing with distilled water. Erythrocyte ghosts generated from 0.1ml human blood were incubated with 50μg EGFP-PLY or 50μg Δ6EGFP-PLY in 1ml 1 × PBS for 30 min at 37°C. The ghost membranes were pelleted, washed × 3 in PBS and were visualized by fluorescence microscopy using a Zeiss Axioscop 20.

The results (not shown) demonstrate that the binding of Δ6 PLY to membranes is substantially the same as that of WT PLY.

Example 15
Analysis of pore formation using Transmission Electron Microscopy.

Electron microscopy was performed as described above (Example 4) for negatively stained horse erythrocyte membranes treated with 0.2mg/ml wild type pneumolysin, 0.2mg/ml W433F PLY, and 0.2mg/ml ΔA146 PLY.

Pores were observed on membranes treated with wt PLY and W433F but not on membranes treated with ΔA146 PLY. Instead, ΔA146 PLY treatment resulted in the formation of long chains, thought to contain self-associated toxin that is unable to oligomerise to form pores. (Data not shown.)

Thus Δ6 PLY retains the membrane-binding properties of wild type PLY but does not form pores in cell membranes.

Example 16
Cytotoxicity of pneumolysin mutants to murine L929 fibroblasts

The cytotoxicity of WT PLY, PLY W433F, and deletion mutants Δ6, Δ7, Δ8 PLY and ΔA146 PLY against murine L929 fibroblasts was determined as described in Example 6. human erythrocytes was compared with that of WT PLY and

The W433F PLY mutant was found to be cytotoxic at 10μg/ml and above, whereas the deletion mutants were non-toxic in this assay (Figure 16).

Example 17

Cytotoxicity of pneumolysin mutant ΔA146 PLY to RBL-2H3 mast cells

The cytotoxicity of ΔA146 PLY against rat RBL-2H3 mast cells was assessed using a degranulation assay.

The assay was carried out as described by Stassen et al (2003) using 10^4 cells/well, incubated with wild type PLY or ΔA146 PLY for 90 minutes.

Release of β-hexosaminidase from mast cell granules was measured, which gives a direct measure of the degranulation of the cells in response to the toxin. ΔA146 PLY did not cause mast cell degranulation (Figure 17).

Example 18

Analysis of murine core body temperature following treatment with wt PLY or ΔA146 PLY

Balb/c mice were implanted with telemetry chips which enable acquisition of core body temperature (Tc). Mice were treated with 1μg wt PLY, 1μg ΔA146 PLY, or saline solution alone.

As shown in Figure 18, Treatment with WT PLY resulted in a severe hypothermic response with Tc dropping to 28°C. This Tc was sustained for 6 hours after which there was an increase in Tc by ~0.6°C/hour and by 24 hours this was similar to the Tc of the control group, though still statistically significant. Treatment with ΔA146 PLY did not result in hypothermia and the median Tc was comparable to the saline control group.

Thus, treatment of mice with WT PLY resulted in a sustained hypothermic response that was not observed following treatment with the same amount of ΔA146 PLY.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention. All references cited herein are expressly incorporated by reference.

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CLAIMS

1. An isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein,

with the proviso that, where the mutant cytolysin is a mutant perfringolysin comprising a substitution or deletion at Y_{181}, the mutant comprises a further mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, wherein the further mutation is capable in isolation of reducing the toxicity of the wild type sequence.

2. The protein of claim 1, wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

3. The protein of claim 1, wherein the mutant protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

4. The protein of claim 3, wherein the mutant protein differs from the wild type by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

5. The protein of claim 4, wherein an amino acid corresponding to alanine 146 of pneumolysin is substituted or deleted.

6. The protein of claim 4, wherein the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

7. The protein of claim 6, wherein amino acids corresponding to valine 144 and proline 145 of pneumolysin are deleted.

8. The protein of claim 6, wherein amino acids corresponding to alanine 146 and arginine 147 of pneumolysin are deleted.

9. The protein of claim 6, wherein amino acids corresponding to methionine 148 and glutamine 149 of pneumolysin are deleted.

10. The protein of claim 6, wherein amino acids corresponding to tyrosine 150 and glutamic acid 151 of pneumolysin are deleted.

11. The protein of any one of claims 1 to 10, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

12. The protein of any one of claims 1 to 11 wherein the mutant cytolysin is a mutant of perfringolysin, intermedilysin or anthrolysin.

13. An immunogenic conjugate comprising:

(a) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and

(b) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein.

14. The immunogenic conjugate of claim 13 wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

15. The immunogenic conjugate of claim 13 wherein the mutant protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

16. The immunogenic conjugate of claim 15 wherein an amino acid corresponding to alanine 146 of pneumolysin is substituted or deleted.

17. The immunogenic conjugate of claim 14, wherein the mutant protein differs from the wild type protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

18. The immunogenic conjugate of claim 17, wherein the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

19. The immunogenic conjugate of claim 18, wherein amino acids corresponding to valine 144 and proline 145 of pneumolysin are deleted.

20. The immunogenic conjugate of claim 18, wherein amino acids corresponding to alanine 146 and arginine 147 of pneumolysin are deleted.

21. The immunogenic conjugate of claim 18, wherein amino acids corresponding to methionine 148 and glutamine 149 of pneumolysin are deleted.

22. The immunogenic conjugate of claim 18, wherein amino acids corresponding to tyrosine 150 and glutamic acid 151 of pneumolysin are deleted.

23. The immunogenic conjugate of any one of claims 13 to 22, wherein the saccharide, oligosaccharide or polysaccharide is derived from the same bacterial species as the wild type cytolysin.

24. The immunogenic conjugate of any one of claims 13 to 23, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

25. The immunogenic conjugate of any one of claims 13 to 24 wherein the mutant cytolysin is a mutant of perfringolysin, intermedilysin or anthrolysin.

26. An isolated and purified nucleic acid sequence comprising a nucleic acid sequence a) encoding a mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids

144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein; or b) which is complementary to a nucleic acid sequence defined in a),

with the proviso that, where the mutant cytolysin is a mutant perfringolysin comprising a substitution or deletion at Y_{181}, the mutant comprises a further mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin (PLY) sequence, wherein the further mutation is capable in isolation of reducing the toxicity of the wild type sequence.

27. The isolated and purified nucleic acid sequence of claim 26, wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

28. The isolated and purified nucleic acid sequence of claim 26, wherein the mutant protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

29. The isolated and purified nucleic acid sequence of claim 28, wherein an amino acid corresponding to alanine 146 of pneumolysin is substituted or deleted.

30. The isolated and purified nucleic acid sequence of claim 28, wherein the mutant protein differs from the wild type protein by the substitution or deletion of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

31. The isolated and purified nucleic acid sequence of claim 30, wherein the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

32. The isolated and purified nucleic acid sequence of claim 31, wherein amino acids corresponding to valine 144 and proline 145 of pneumolysin are deleted.

33. The isolated and purified nucleic acid sequence of claim 31, wherein amino acids corresponding to alanine 146 and arginine 147 of pneumolysin are deleted.

34. The isolated and purified nucleic acid sequence of claim 31, wherein amino acids corresponding to methionine 148 and glutamine 149 of pneumolysin are deleted.

35. The isolated and purified nucleic acid sequence of claim 31, wherein amino acids corresponding to tyrosine 150 and glutamic acid 151 of pneumolysin are deleted.

36. The isolated and purified nucleic acid sequence of any one of claims 26 to 35, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

37. The isolated and purified nucleic acid sequence of any one of claims 26 to 36 wherein the mutant cytolysin is a mutant of perfringolysin, intermedilysin or anthrolysin.

38. A recombinant expression vector comprising an isolated and purified nucleic acid sequence comprising a nucleic acid sequence of any one of claims 26 to 37.

39. A recombinant host cell transformed, transfected or infected with a recombinant expression vector of claim 38.

40. A method of producing an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein, which comprises: a) transforming, transfecting or infecting a host cell with the recombinant expression vector of claim 38 and culturing the host cell under conditions which permit the expression of said mutant protein by the host cell; and b) recovering the mutant protein from the culture.

41. An immunogenic composition which comprises: a) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

42. The immunogenic composition of claim 41, wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

43. The immunogenic composition of claim 41, wherein the mutant protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

44. The immunogenic composition of claim 43, wherein an amino acid corresponding to alanine 146 of pneumolysin is substituted or deleted.

45. The immunogenic composition of claim 43, wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

46. The immunogenic composition of claim 45, wherein the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type sequence.

47. The immunogenic composition of claim 46, wherein amino acids corresponding to valine 144 and proline 145 of pneumolysin are deleted.

48. The immunogenic composition of claim 46, wherein amino acids corresponding to alanine 146 and arginine 147 of pneumolysin are deleted.

49. The immunogenic composition of claim 46, wherein amino acids corresponding to methionine 148 and glutamine 149 of pneumolysin are deleted.

50. The immunogenic composition of claim 46, wherein amino acids corresponding to tyrosine 150 and glutamic acid 151 of pneumolysin are deleted.

51. The immunogenic composition of any one of claims 41 to 50, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the

regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

52. The immunogenic composition of any one of claims 41 to 51, wherein the mutant cytolysin is a mutant of perfringolysin, intermedilysin or anthrolysin.

53. An immunogenic composition which comprises: a) an immunogenic conjugate comprising:

(i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and

(ii) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein; and

b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

54. The immunogenic composition of claim 53, wherein the mutant protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence.

55. The immunogenic composition of claim 53, wherein the mutant protein differs from the wild type protein by the presence of a mutation within the region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

56. The immunogenic composition of claim 55, wherein the mutant protein differs from the wild type protein by the substitution or deletion of one or more amino acids within the

region corresponding to amino acids 144 to 151 of the wild type pneumolysin sequence.

57. The immunogenic composition of claim 56, wherein an amino acid corresponding to alanine 146 of pneumolysin is substituted or deleted.

58. The immunogenic composition of claim 56, wherein the mutant protein differs from the wild type protein by the deletion of two adjacent amino acids within the region corresponding to amino acids 144 to 151 of the wild type sequence.

59. The immunogenic composition of claim 58, wherein amino acids corresponding to valine 144 and proline 145 of pneumolysin are deleted.

60. The immunogenic composition of claim 58, wherein amino acids corresponding to alanine 146 and arginine 147 of pneumolysin are deleted.

61. The immunogenic composition of claim 58, wherein amino acids corresponding to methionine 148 and glutamine 149 of pneumolysin are deleted.

62. The immunogenic composition of claim 58, wherein amino acids corresponding to tyrosine 150 and glutamic acid 151 of pneumolysin are deleted.

63. The immunogenic composition of any one of claims 53 to 62, wherein the saccharide, oligosaccharide or polysaccharide is derived from the same bacterial species as the wild type cytolysin.

64. The immunogenic composition of claim 63, wherein there are a plurality of serotypes of said species.

65. The immunogenic composition of any one of claims 53 to 64, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions corresponding to amino acids 257-297, 367-397 or 424-437 of the wild type pneumolysin sequence.

66. The immunogenic composition of any one of claims 53 to 65 wherein the mutant cytolysin is a mutant of perfringolysin, intermedilysin or anthrolysin.

67. A method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition which comprises: a) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

68. A method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition which comprises: a) an immunogenic conjugate comprising:

(i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and

(ii) an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is

reduced relative to that of the wild-type cytolysin protein; and

b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

69. Use of a mutant cytolysin protein or immunogenic conjugate thereof in the preparation of an immunogenic composition for the prophylaxis or treatment of bacterial infection, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein.

70. An isolated mutant cytolysin protein or immunogenic conjugate thereof for use in a method of medical treatment, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein.

71. A method of preparation of an immunogenic composition, the method comprising the steps of:

providing an isolated mutant cytolysin protein, wherein the mutant cytolysin protein differs from the wild type cytolysin protein by the presence of a mutation within the region corresponding to amino acids 144 to 161 of the wild type pneumolysin sequence, such that the toxicity of the mutant cytolysin protein is reduced relative to that of the wild-type cytolysin protein; and

conjugating the mutant cytolysin protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein.

72. The method of claim 61, wherein the mutant protein is conjugated to a polysaccharide from the same bacterial species as the wild type cytolysin.

73. A method of screening candidate mutant cytolysin proteins for suitability for use in immunogenic compositions, the method comprising the steps of:

providing a mutant cytolysin protein;

testing the mutant cytolysin protein for haemolytic activity;

testing the mutant cytolysin protein for oligomerisation activity; and

comparing the haemolytic and oligomerisation activity of the mutant cytolysin protein with those of a non-mutant protein.

A. Pneumolysin from *Streptococcus pneumoniae*
Length: 471 AA g.i. 47403 (X52474)

```
1   MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
51  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
101 IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
151 EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
201 TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
251 ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
301 RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
351 VETKVTAYRN GDLLLDHSGA YVAQYYITWN ELSYDHQGKE VLTPKAWDRN
401 GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
451 RTISIWGTTL YPQVEDKVEN D
```

B. Perfringolysin O from *Clostridium perfringens*
Length: 500 AA g.i. 144884 (AAA23270)

```
1   MIRFKKTKLI ASIAMALCLF SQPVISFSKD ITDKNQSIDS GISSLSYNRN
51  EVLASNGDKI ESFVPKEGKK TGNKFIVVER QKRSLTTSPV DISIIDSVND
101 RTYPGALQLA DKAFVENRPT ILMVKRKPIN INIDLPGLKG ENSIKVDDPT
151 YGKVSGAIDE LVSKWNEKYS STHTLPARTQ YSESMVYSKS QISSALNVNA
201 KVLENSLGVD FNAVANNEKK VMILAYKQIF YTVSADLPKN PSDLFDDSVT
251 FNDLKQKGVS NEAPPLMVSN VAYGRTIYVK LETTSSSKDV QAAFKALIKN
301 TDIKNSQQYK DIYENSSFTA VVLGGDAQEH NKVVTKDFDE IRKVIKDNAT
351 FSTKNPAYPI SYTSVFLKDN SVAAVHNKTD YIETTSTEYS KGKINLDHSG
401 AYVAQFEVAW DEVSYDKEGN EVLTHKTWDG NYQDKTAHYS TVIPLEANAR
451 NIRIKARECT GLAWEWWRDV ISEYDVPLTN NINVSIWGTT LYPGSSITYN
```

Figure 1

C. Intermedilysin from *Streptococcus intermedius*

Length: 532 AA gi: 6729344 (NCBI - BAA89790)

```
1   MKTKQNIARK LSRVVLLSTL VLSSAAPISA AFAETPTKPK AAQTEKKTEK
51  KPENSNSEAA KKALNDYIWG LQYDKLNILT HQGEKLKNHS SREAFHRPGE
101 YVVIEKKKQS ISNATSKLSV SSANDDRIFP GALLKADQSL LENLPTLIPV
151 NRGKTTISVN LPGLKNGESN LTVENPSNST VRTAVNNLVE KWIQNYSKTH
201 AVPARMQYES ISAQSMSQLQ AKFGADFSKV GAPLNVDFSS VHKGEKQVFI
251 ANFRQVYYTA SVDSPNSPSA LFGSGITPTD LINRGVNSKT PPVYVSNVSY
301 GRAMYVKFET TSKSTKVQAA IDAVVKGAKL KAGTEYENIL KNTKITAVVL
351 GGNPGEASKV ITGNIDTLKD LIQKGSNFSA QSPAVPISYT TSFVKDNSIA
401 TIQNNTDYIE TKVTSYKDGA LTLNHDGAFV ARFYVYWEEL GHDADGYETI
451 RSRSWSGNGY NRGAHYSTTL RFKGNVRNIR VKVLGATGLA WEPWRLIYSK
501 NDLPLVPQRN ISTWGTTLHP QFEDKVVKDN TD
```

D. Alveolysin from *Bacillus alvei*

Length: 501 AA gi: 142473 (NCBI - AAA22224)

```
1   MKKKSNHLKG RKVLVSLLVS LQVFAFASIS SAAPTEPNDI DMGIAGLNYN
51  RNEVLAIQGD QISSFVPKEG IQSNGKFIVV ERDKKSLTTS PVDISIVDSI
101 TNRTYPGAIQ LANKDFADNQ PSLVMAARKP LDISIDLPGL KNENTISVQN
151 PNYGTVSSAI DQLVSTWGEK YSSTHTLPAR LQYAESMVYS QNQISSALNV
201 NAKVLNGTLG IDFNAVANGE KKVMVAAYKQ IFYTVSAGLP NNPSDLFDDS
251 VTFAELARKG VSNEAPPLMV SNVAYGRTIY VKLETTSKSN DVQTAFKLLL
301 NNPSIQASGQ YKDIYENSSF TAVVLGGDAQ THNQVVTKDF NVIQSVIKDN
351 AQFSSKNPAY PISYTSVFLK DNSIAAVHNN TEYIETKTTE YSKGKIKLDH
401 SGAYVAQFEV YWDEFSYDAD GQEIVTRKSW DGNWRDRSAH FSTEIPLPPN
451 AKNIRIFARE CTGLAWEWWR TVVDEYNVPL ASDINVSIWG TTLYPKSSIT
501 H
```

Figure 1 cont'd

E. Anthrolysin from *Bacillus anthracis*
Length: 512 AA gi: 21397375 (NCBI - NC003995)

```
1   MIFLNIKKNT KRRKFLACLL VSLCTIHYSS ISFAETQAGN ATGAIKNASD
51  INTGIANLKY DSRDILAVNG DKVESFIPKE SINSNGKFVV VEREKKSLTT
101 SPVDILIIDS VVNRTYPGAV QLANKAFADN QPSLLVAKRK PLNISIDLPG
151 MRKENTITVQ NPTYGNVAGA VDDLVSTWNE KYSTTHTLPA RMQYTESMVY
201 SKSQIASALN VNAKYLDNSL NIDFNAVANG EKKVMVAAYK QIFYTVSAEL
251 PNNPSDLFDN SVTFDELTRK GVSNSAPPVM VSNVAYGRTV YVKLETTSKS
301 KDVQAAFKAL LKNNSVETSG QYKDIFEEST FTAVVLGGDA KEHNKVVTKD
351 FNEIRNIIKD NAELSFKNPA YPISYTSTFL KDNATAAVHN NTDYIETTTT
401 EYSSAKMTLD HYGAYVAQFD VSWDEFTFDQ NGKEVLTXKX WEGSGKDKTA
451 HYSTVIPLPP NSKNIKIVAR ECTGLAWEWW RTIINEQNVP LTNEIKVSIG
501 GTTLYPTATI SH
```

F. Putative Cereolysin from *Bacillus cereus*
Length 500a.a gi:418066 (NCBI - D21270)

```
1   MKNFKGRKFL TCVLVSLCTL NYSSISFAET QAGHANDITK NASSIDTGIG
51  NLTYNNQEVL AVNGDKVESF VPKESINSNG KFVVVDVRKN HLQRHQSIFR
101 LLDSVANRTY PGAVQLANKA FADNQPSLLV AKRKPLNISI DLPGMRKENT
151 ITVQNPTYGN VAGAVDDLVS TWNEKYSATH TLPARMQYTE SMVYSKAQIA
201 SALNVNAKYL DNSLNIDFNA VANGEKKVMV AAYKQIFYTV SAELPNNPSD
251 LFDNSVTFGE LTRKGVSNSA PPVMVSNVAY GRTVYVKLET TSKSKDVQAA
301 FKALLKNNSV ETSGQYKDIF EESTFTAVVL GGDAKEHNKV VTKDFNEIRN
351 IIKDNAELSF KNPAYPISYT STFLKDNATA AVHNNTDYIE TTTTEYSSAK
401 MTLDHYGAYV AQFDVSWDGF TFDQNGKEIL THKTWEGSGK DKTAHYSTVI
451 PLPPNSKNIK IVARECTGLA WEWWRTIIKM NKMFH
```

G. Ivanolysin O from *Listeria ivanovii*
Length: 528 AA gi: 7649482 (CAA42995)

Figure 1 cont'd

1 MKKIMLLLMT LLLVSLPLAQ EAQADASVYS YQGIISHMAP PASPPAKPKT
51 PVEKKNAAQI DQYIQGLDYD KNNILVYDGE AVKNVPPKAG YKEGNQYIVV
101 EKKKKSINQN NADIQVINSL ASLTYPGALV KANSELVENQ PDVLPVKRDS
151 VTLSIDLPGM VNHDNEIVVQ NATKSNINDG VNTLVDRWNN KYSEEYPNIS
201 AKIDYDQEMA YSESQLVAKF GAAFKAVNNS LNVNFGAISE GKVQEEVINF
251 KQIYYTVNVN EPTSPSRFFG KSVTKENLQA LGVNAENPPA YISSVAYGRD
301 IFVKLSTSSH STRVKAAFDA AFKGKSVKGD TELENIIQNA SFKAVIYGGS
351 AKDEVEIIDG DLSKLRDILK QGANFDKKNP GVPIAYTTNF LKDNQLAVVK
401 NNSEYIETTS KAYSDGKINL DHSGAYVARF NVTWDEVSYD ANGNEVVEHK
451 KWSENDKDKL AHFTTSIYLP GNARNINIHA KECTGLAWEW WRTVVDDRNL
501 PLVKNRNVCI WGTTLYPAYS DTVDNPIK

H. Pyolysin from *Arcanobacterium pyogenes*
Length: 534 AA gi: 2252800 (AAC45754)

1 MKRKAFASLV ASVVAAATVT MPTASFAAGL GNSSGLTDGL SAPRVSISPM
51 DKVDLKSAQE TDETSVDKYI RGLEYDPSGV LAVKGESIEN VPVTKDQLKD
101 GTYTVFKHER KSFNNLRSDI SAFDANNAHV YPGALVLANK DLAKGSPTSI
151 GIARAPQTVS VDLPGLVDGK SKVVINNPTK SSVTQGMNGL LDGWIQRNSK
201 YPDHAAKIFY DETMVTSKRQ LEAKFGLGFE KVSAKLNVDF DAIHKRERQV
251 AIASFKQIYY TASVDTPTSP HSVFGPNVTA QDLKDRGVNN KNPLGYISSV
301 SYGRQIFVKL ETTSTSNDVQ AAFSGLFKAK FGNLSTEFKA KYADILNKTR
351 ATVYAVGGSA RGGVEVATGN IDALKKIIKE ESTYSTKVPA VPVSYSVNFL
401 KDNQLAAVRS SGDYIETTAT TYKSGEITFR HGGGYVAKFG LKWDEISYDP
451 QGKEIRTPKT WSGNWVGRTL GFRETIQLPA NARNIHVEAG EATGLAWDPW
501 WTVINKKNLP LVPHREIVLK GTTLNPWVEE NVKS

I. Seeligeriolysin O from *Listeria seeligeri*
Length: 530 AA gi: 44145 (CAA42996)

1 MKIFGLVIMS LLFVSLPITQ QPEARDVPAY DRSEVTISPA ETPESPPATP
51 KTPVEKKHAE EINKYIWGLN YDKNSILVYQ GEAVTNVPPK KGYKDGSEYI
101 VVEKKKKGIN QNNADISVIN AISSLTYPGA LVKANRELVE NQPNVLPVKR

Figure 1 cont'd

151 DSLTLSVDLP GMTKKDNKIF VKNPTKSNVN NAVNTLVERW NDKYSKAYPN
201 INAKIDYSDE MAYSESQLIA KFGTAFKAVN NSLNVNFEAI SDGKVQEEVI
251 SFKQIYYNIN VNEPTSPSKF FGGSVTKEQL DALGVNAENP PAYISSVAYG
301 RQVYVKLSSS SHSNKVKTAF EAAMSGKSVK GDVELTNIIK NSSFKAVIYG
351 GSAKEEVEII DGNLGELRDI LKKGSTYDRE NPGVPISYTT NFLKDNDLAV
401 VKNNSEYIET TSKSYTDGKI NIDHSGGYVA QFNISWDEVS YDENGNEIKV
451 HKKWGENYKS KLAHFTSSIY LPGNARNINI YARECTGLFW EWWRTVIDDR
501 NLPLVKNRNV SIWGTTLYPR HSNNVDNPIQ

J. Streptolysin O from *S. pyogenes*
Length: 574 AA gi: 19747435 (AAL96968)

1 MKDMSNKKTF KKYSRVAGLL TAALIIGNLV TANAESNKQN TASTETTTTN
51 EQPKPESSEL TTEKAGQKTD DMLNSNDMIK LAPKEMPLES AEKEEKKSED
101 KKKSEEDHTE EINDKIYSLN YNELEVLAKN GETIENFVPK EGVKKADKFI
151 VIERKKKNIN TTPVDISIID SVTDRTYPAA LQLANKGFTE NKPDAVVTKR
201 NPQKIHIDLP GMGDKATVEV NDPTYANVST AIDNLVNQWH DNYSGGNTLP
251 ARTQYTESMV YSKSQIEAAL NVNSKILDGT LGIDFKSISK GEKKVMIAAY
301 KQIFYTVSAN LPNNPADVFD KSVTFKELQR KGVSNEAPPL FVSNVAYGRT
351 VFVKLETSSK SNDVEAAFSA ALKGTDVKTN GKYSDILENS SFTAVVLGGD
401 AAEHNKVVTK DFDVIRNVIK DNATFSRKNP AYPISYTSVF LKNNKIAGVN
451 NRTEYVETTS TEYTSGKINL SHRGAYVAQY EILWDEINYD DKGKEVITKR
501 RWDNNWYSKT SPFSTVIPLG ANSRNIRIMA RECTGLAWEW WRKVIDERDV
551 KLSKEINVNI SGSTLSPYGS ITYK

K. Suilysin from *Streptococcus suis*
Length: 497 AA gi: 30088598 (AAN34600)

1 MRKSSHLILS SIVSLALVGV TPLSVLADSK QDINQYFQSL TYEPQEILTN
51 EGEYIDNPPA TTGMLENGRF VVLRREKKNI TNNSADIAVI DAKAANIYPG
101 ALLRADQNLL DNNPTLISIA RGDLTLSLNL PGLANGDSHT VVNSPTRSTV

Figure 1 cont'd

151 RTGVNNLLSK WNNTYAGEYG NTQAELQYDE TMAYSMSQLK TKFGTSFEKI
201 AVPLDINFDA VNSGEKQVQI INFKQIYYTV SVDEPESPSK LFAEGTTVED
251 LQRNGITDEV PPVYVSSVSY GRSMFIKLET SSRSTQVQAA FKAAIKGVDI
301 SGNAEYQDIL KNTSFSAYIF GGDAGSAATV VSGNIETLKK IIEEGARYGK
351 LNPGVPISYS TNFVKDNRPA QILSNSEYIE TTSTVHNSSA LTLDHSGAYV
401 AKYNITWEEV SYNEAGEEVW EPKAWDKNGV NLTSHWSETI QIPGNARNLH
451 VNIQECTGLA WEWWRTVYDK DLPLVGQRKI TIWGTTLYPQ YADEVIE

L. Tetanolysin from *Clostridium tetani*
Length: 527 AA gi: 28211522 (NP782466)

1 MNKNVLKFVS RSLLIFSMTG LISNYNSSNV LAKGNVEEHS LINNGQVVTS
51 NTKCNLAKDN SSDIDKNIYG LSYDPRKILS YNGEQVENFV PAEGFENPDK
101 FIVVKREKKS ISDSTADISI IDSINDRTYP GAIQLANRNL MENKPDIISC
151 ERKPITISVD LPGMAEDGKK VVNSPTYSSV NSAINSILDT WNSKYSSKYT
201 IPTRMSYSDT MVYSQSQLSA AVGCNFKALN KALNIDFDSI FKGEKKVMLL
251 AYKQIFYTVS VDPPNRPSDL FGDSVTFDEL ALKGINNNNP PAYVSNVAYG
301 RTIYVKLETT SKSSHVKAAF KALINNQDIS SNAEYKDILN QSSFTATVLG
351 GGAQEHNKII TKDFDEIRNI IKNNSVYSPQ NPGYPISYTT TFLKDNSIAS
401 VNNKTEYIET TATEYTNGKI VLDHSGAYVA QFQVTWDEVS YDEKGNEIVE
451 HKAWEGNNRD RTAHFNTEIY LKGNARNISV KIRECTGLAW EWWRTIVDVK
501 NIPLAKERTF YIWGTTLYPK TSIETKM

M. Listeriolysin O from *Listeria monocytogenes*
Length: 529 AA gi: 7595976 (AAF64524)

1 MKKIMLVFIT LILISLPIAQ QTEAKDASAF HKEDLISSMA PPTSPPASPK
51 TPIEKKHADE IDKYIQGLDY NKNNVLVYHG DAVTNVPPRK GYKDGNEYIV
101 VEKKKKSINQ NNADIQVVNA ISSLTYPGAL VKANSELVEN QPDVLPVKRD
151 SLTLSIDLPG MTNQDNKIVV KNATKSNVNN AVNTLVERWN EKYAQAYPNV
201 SAKIDYDDEM AYSESQLIAK FGTAFKAVNN SLNVNFGAIS EGKMQEEVIS

Figure 1 cont'd

```
251 FKQIYYNVNV NEPTRPSRFF GKAVTKEQLQ ALGVNAENPP AYISSVAYGR
301 QVYLKLSTNS HSTKVKAAFD AAVSGKSVSG DVELTNIIKN SSFKAVIYGG
351 SAKDEVQIID GNLGDLRDIL KKGATFNRET PGVPIAYTTN FLKDNELAVI
401 KNNSEYIETT SKAYTDGKIN IDHSGGYVAQ FNISWDEINY DPEGNEIVQH
451 KNWSENNKSK LAHFTSSIYL PGNARNINVY AKECTGLAWE WWRTVIDDRN
501 LPLVKNRNIS IWGTTLYPKY SNSVDNPIE
```

Figure 1 cont'd

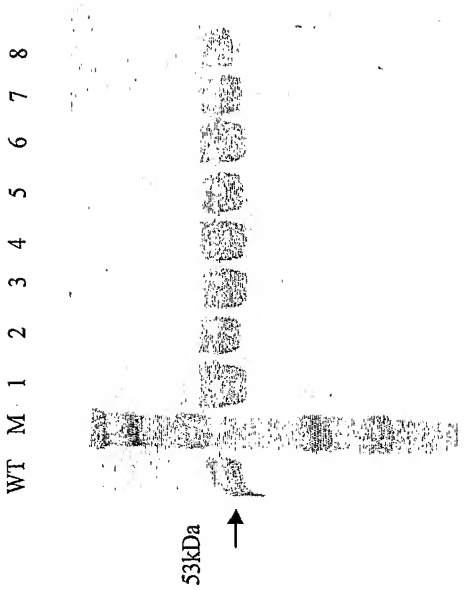

Figure 2

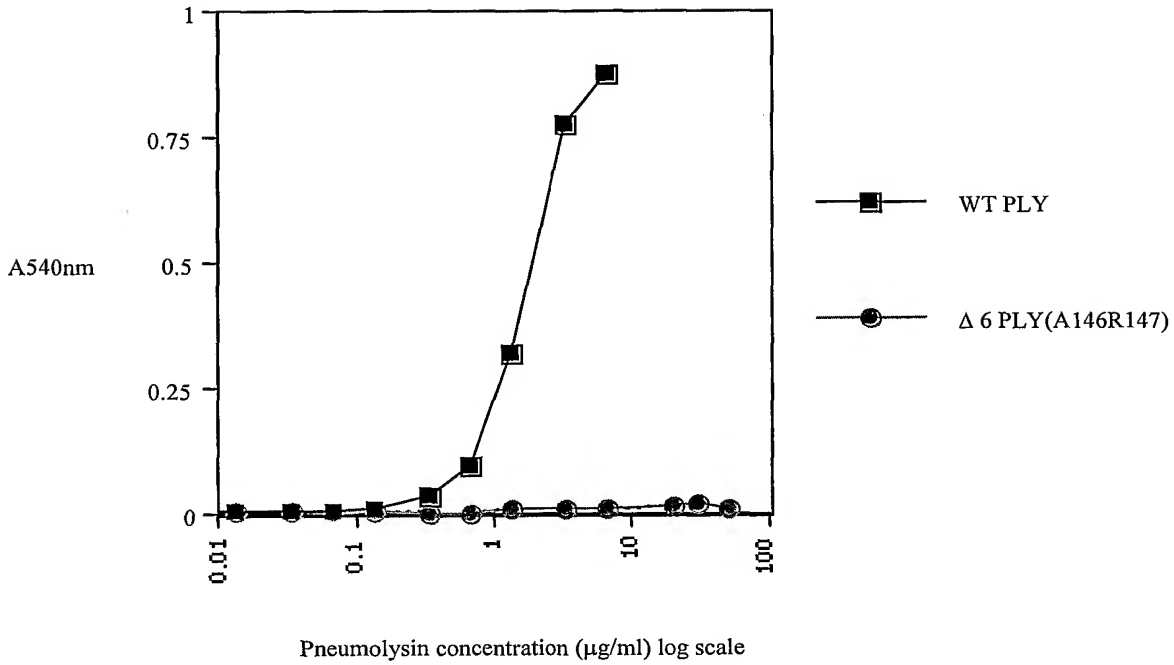
A540nm
1
0.75
0.5
0.25
0
0.01
0.1
1
10
100
Pneumolysin concentration (μg/ml) log scale
WT PLY
Δ 6 PLY(A146R147)

Figure 3

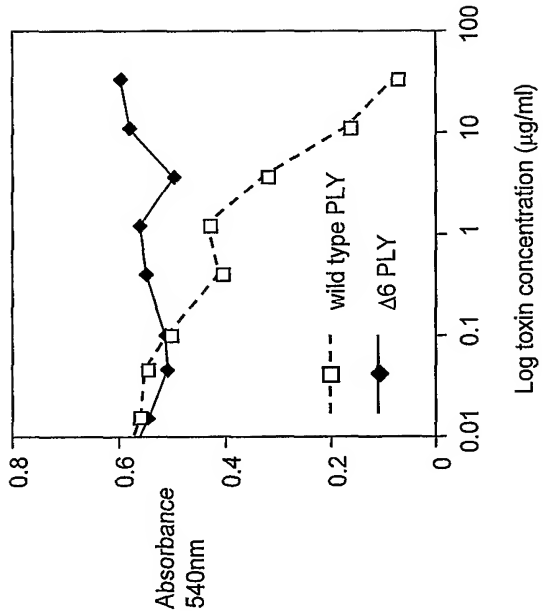
Absorbance 540nm
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
Log toxin concentration (µg/ml)
wild type PLY
Δ6 PLY

Figure 4

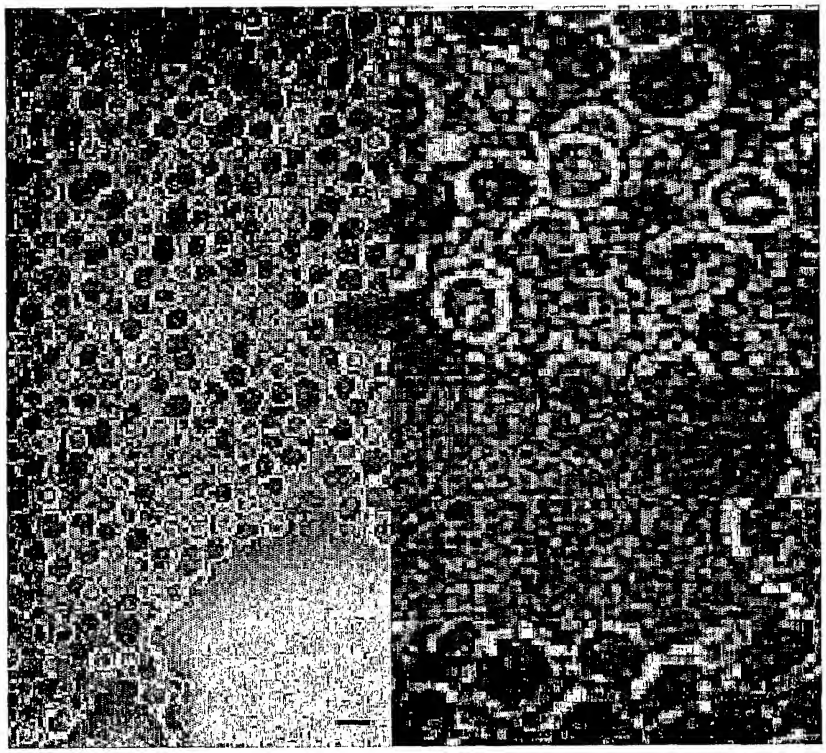

Figure 5

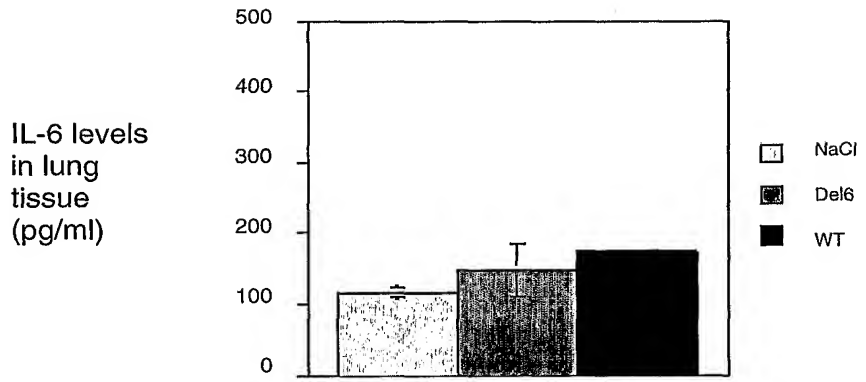
IL-6 levels in lung tissue (pg/ml)
500
400
300
200
100
0
NaCl
Del6
WT

Figure 6

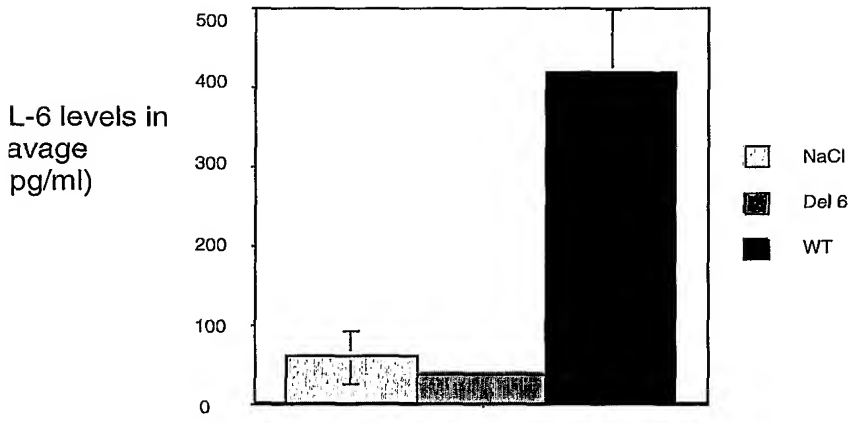

Figure 7

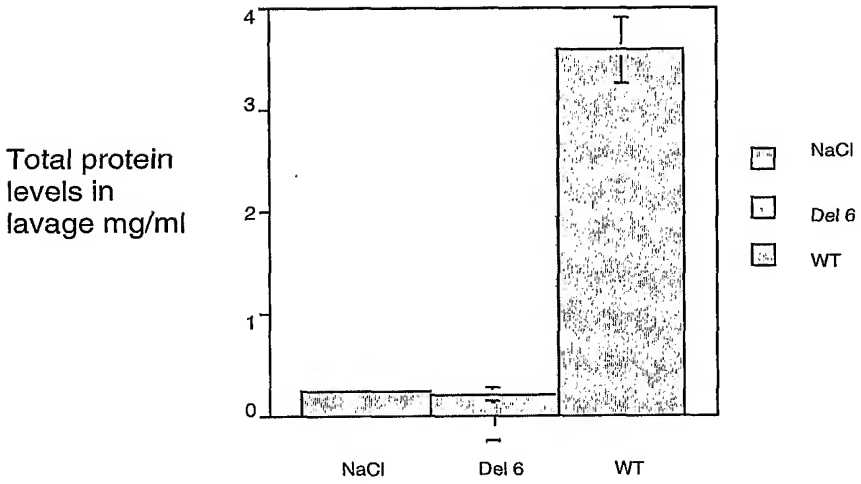
Total protein levels in lavage mg/ml
4
3
2
1
0
NaCl
Del 6
WT
NaCl
Del 6
WT

Figure 8

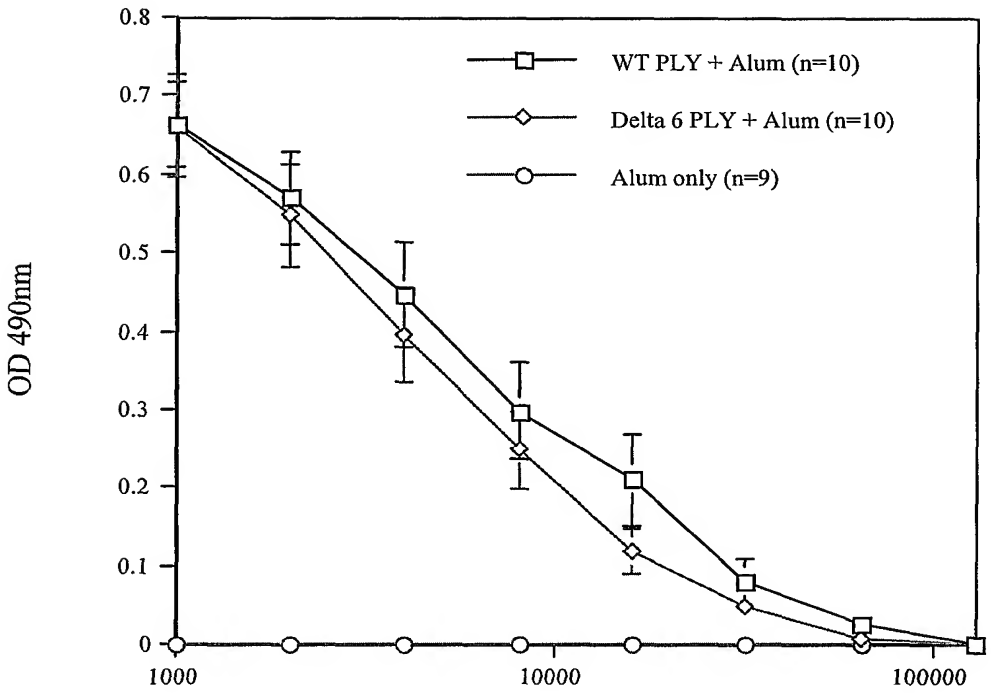
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 490nm
WT PLY + Alum (n=10)
Delta 6 PLY + Alum (n=10)
Alum only (n=9)
1000
10000
100000
1/Dilution

Figure 9

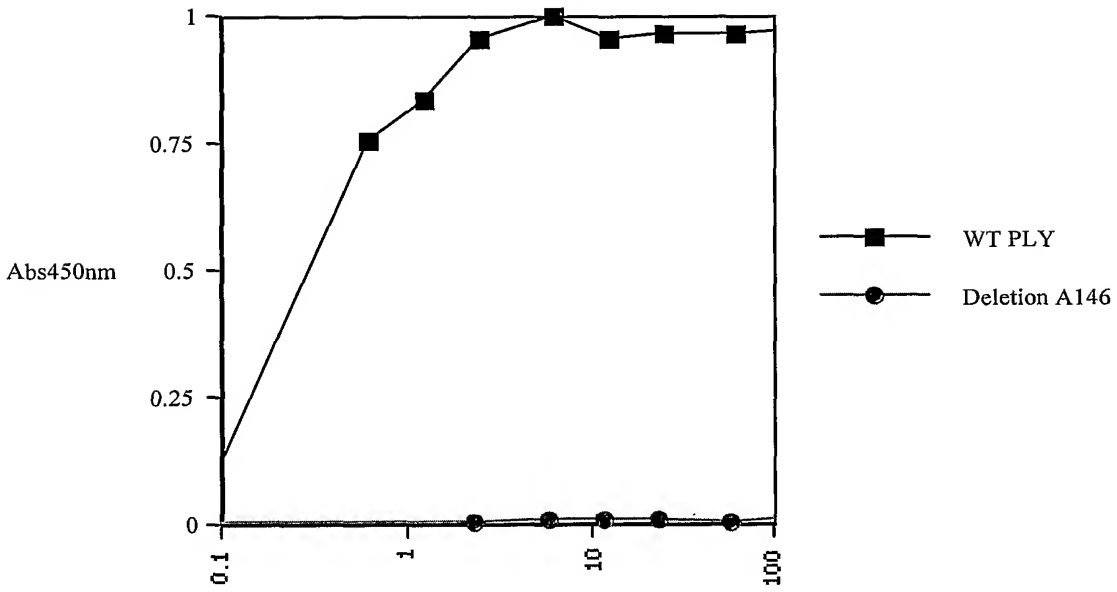

Figure 10

1. Marker
2. wild type PFO
3. Δ6 PFO
4. wild type PLY

Figure 11

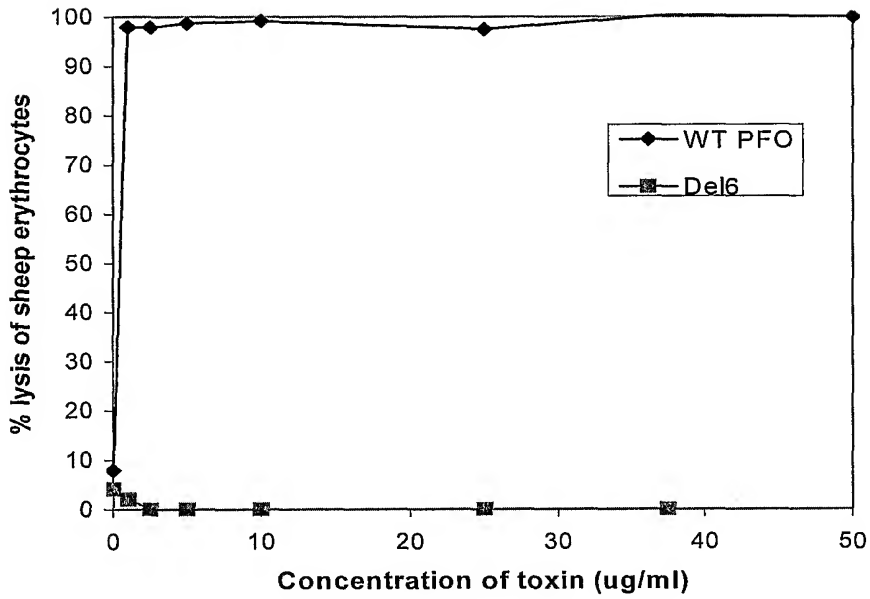
100
90
80
70
60
50
40
30
20
10
0
% lysis of sheep erythrocytes
0
10
20
30
40
50
Concentration of toxin (ug/ml)
WT PFO
Del6

Figure 12

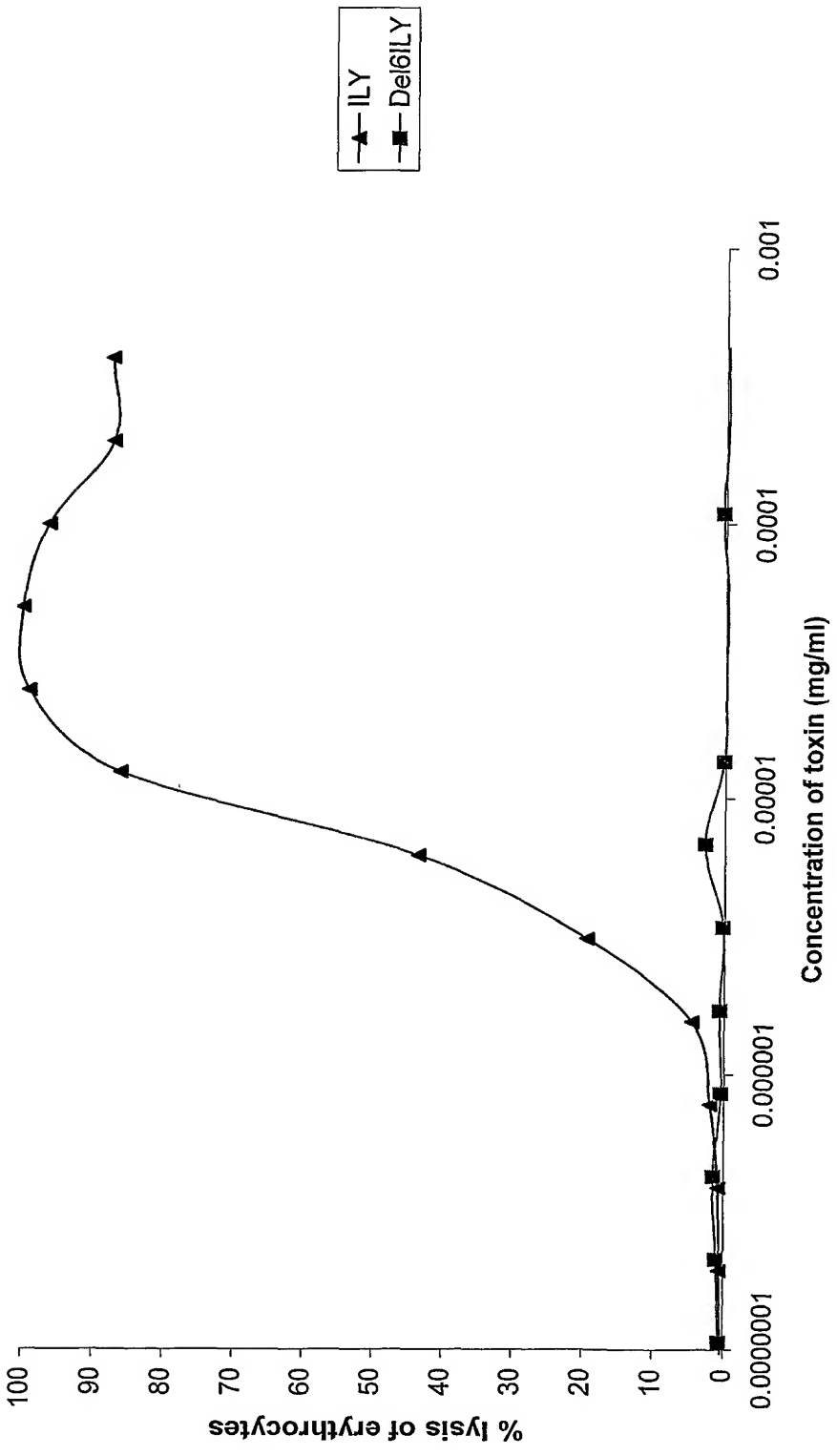
ILY
Del6ILY
% lysis of erythrocytes
100
90
80
70
60
50
40
30
20
10
0
0.0000001
0.000001
0.00001
0.0001
0.001
Concentration of toxin (mg/ml)

Figure 13

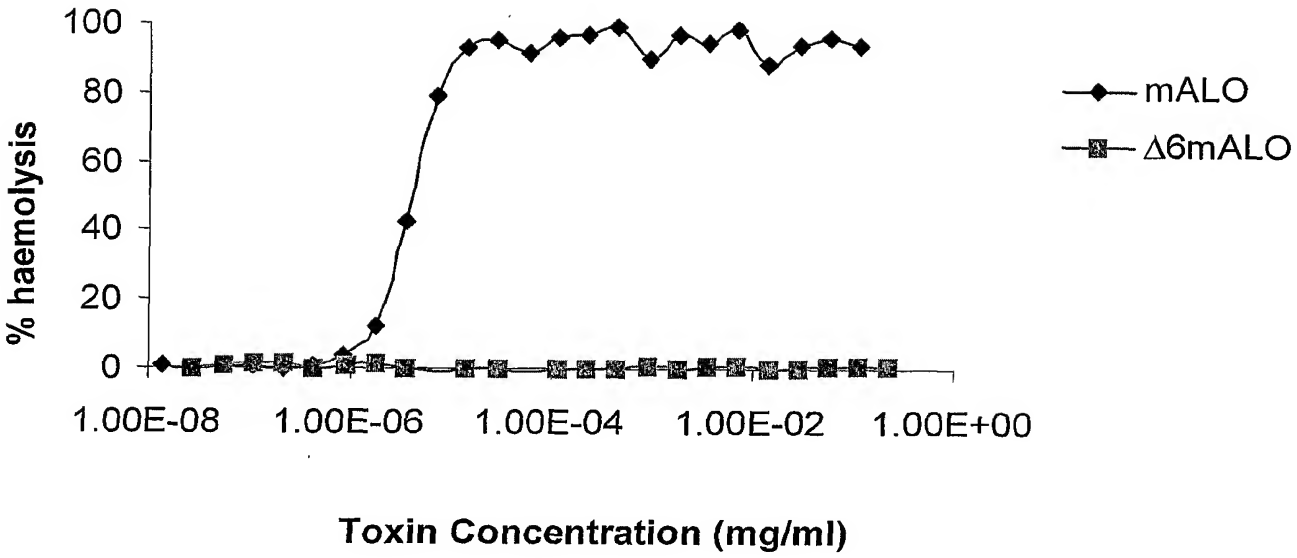
100
80
60
40
20
0
% haemolysis
1.00E-08
1.00E-06
1.00E-04
1.00E-02
1.00E+00
Toxin Concentration (mg/ml)
mALO
Δ6mALO

Figure 14

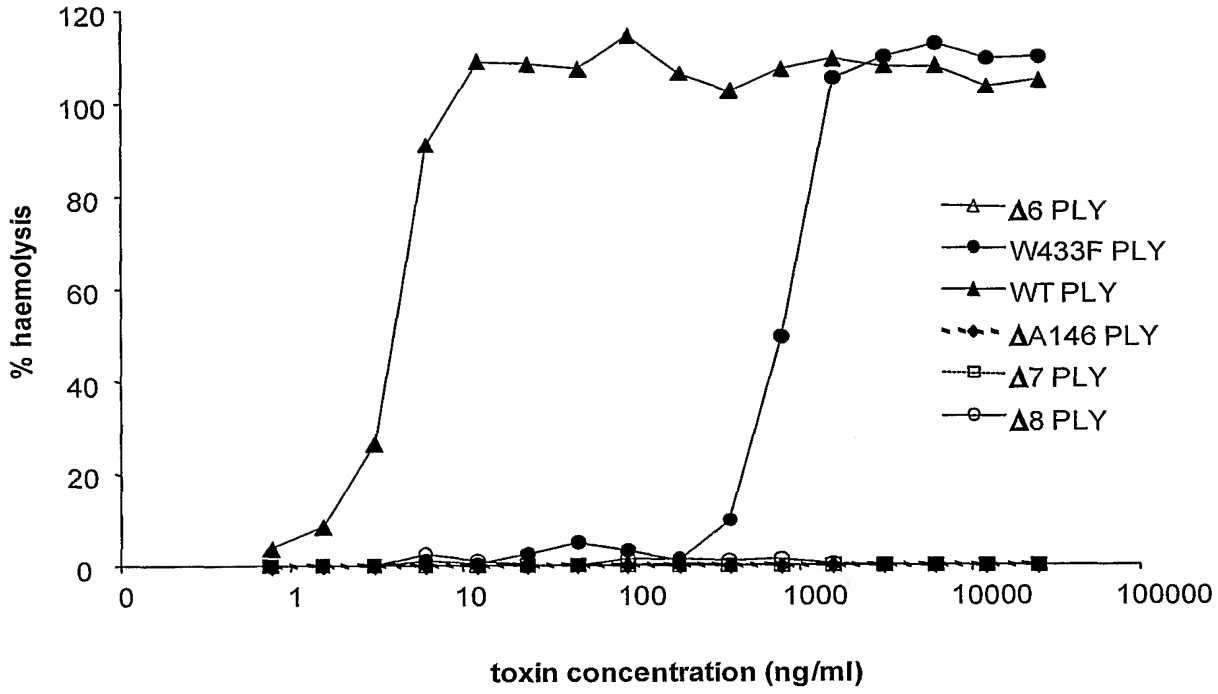
120
100
80
60
40
20
0
% haemolysis
0
1
10
100
1000
10000
100000
toxin concentration (ng/ml)
Δ6 PLY
W433F PLY
WT PLY
ΔA146 PLY
Δ7 PLY
Δ8 PLY

Figure 15

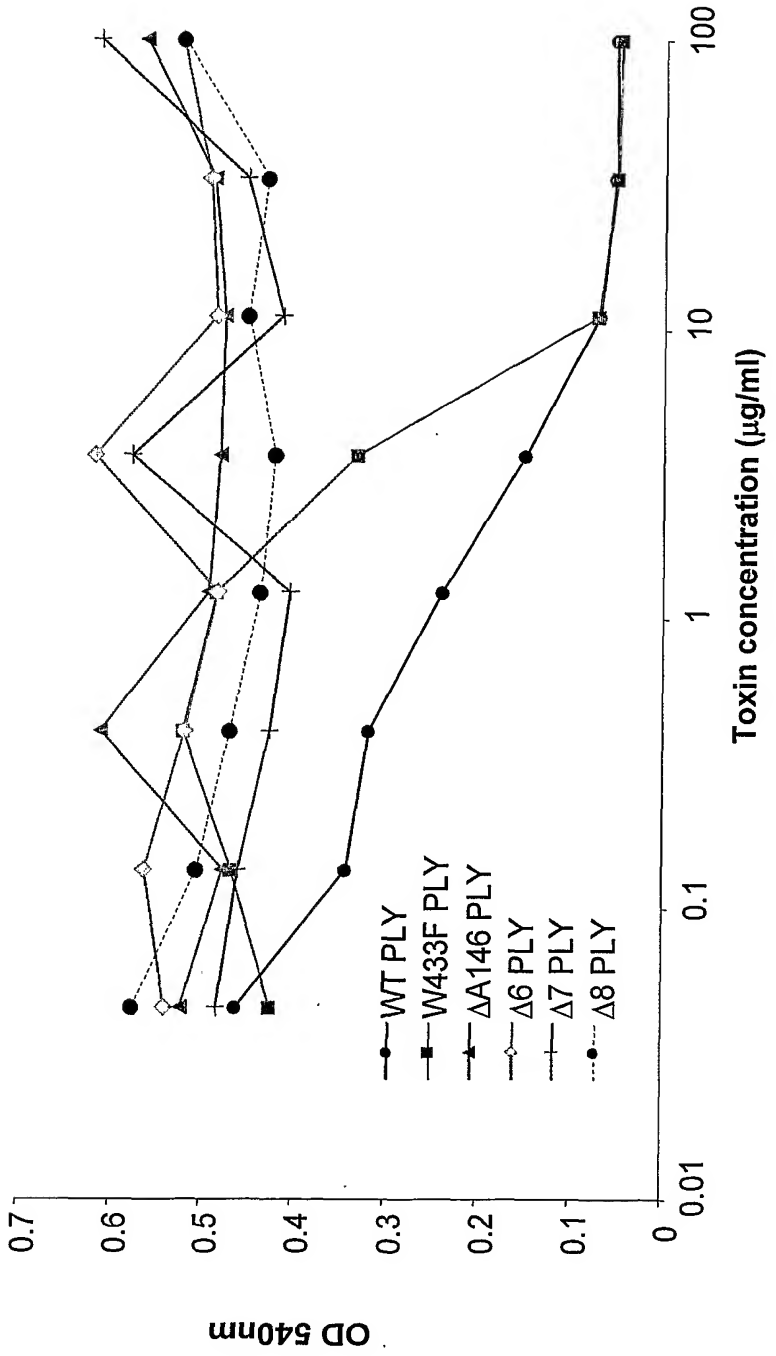
OD 540nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.01
0.1
1
10
100
Toxin concentration (µg/ml)
WT PLY
W433F PLY
ΔA146 PLY
Δ6 PLY
Δ7 PLY
Δ8 PLY

Figure 16

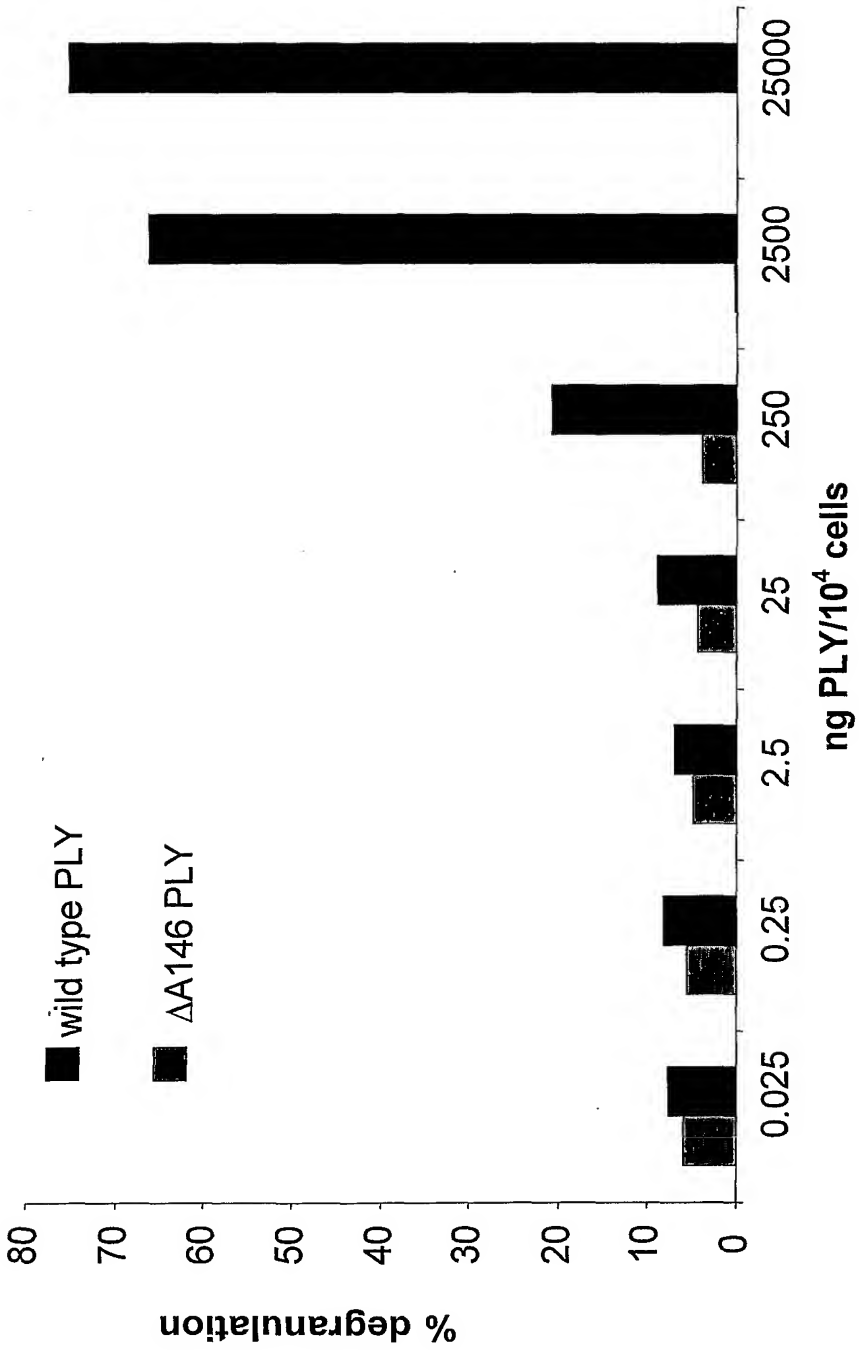
% degranulation
0
10
20
30
40
50
60
70
80
wild type PLY
ΔA146 PLY
0.025
0.25
2.5
25
250
2500
25000
ng PLY/10⁴ cells

Figure 17

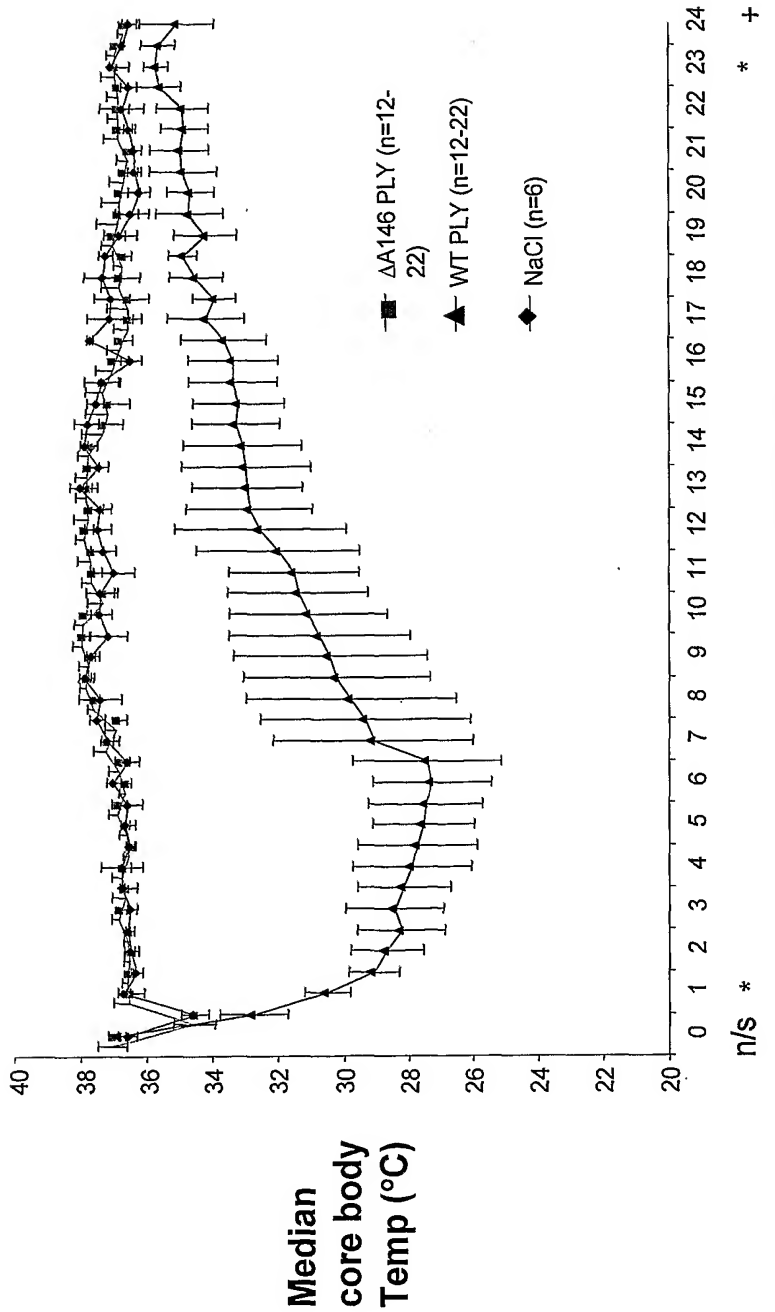
Median core body Temp (°C)
40
38
36
34
32
30
28
26
24
22
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (h)
n/s *
* +
ΔA146 PLY (n=12-22)
WT PLY (n=12-22)
NaCl (n=6)
* P<0.01; + P<0.05

Figure 18

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB2005/001774

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/195 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, Sequence Search, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2001/014332 A1 (MINETTI CONCEICAO ET AL) 16 August 2001 (2001-08-16) abstract page 6, left-hand column; table 1 page 21, right-hand column; claim 24	1-73
X	HILL J ET AL: "AMINO ACIDS IN PNEUMOLYSIN IMPORTANT FOR HEMOLYTIC ACTIVITY IDENTIFIED BY RANDOM MUTAGENESIS" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 62, no. 2, February 1994 (1994-02), pages 757-758, XP001194645 ISSN: 0019-9567 page 758, left-hand column; table 1	1-73

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2005	02/09/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Grötzinger, T

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INTERNATIONAL SEARCH REPORT

Intern I Application No
PCT/GB2005/001774

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOTZE EILEEN M ET AL: "Monomer-monomer interactions drive the prepore to pore conversion of a beta-barrel-forming cholesterol-dependent cytolysin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 13, 29 March 2002 (2002-03-29), pages 11597-11605, XP002341458 ISSN: 0021-9258 cited in the application abstract page 11599, left-hand column, line 2 - line 6	1-73
P,X	RAMACHANDRAN RAJESH ET AL: "Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment" NATURE STRUCTURAL & MOLECULAR BIOLOGY, vol. 11, no. 8, August 2004 (2004-08), pages 697-705, XP002341459 ISSN: 1545-9993 published online on 4 July 2004 page 702, right-hand column, line 20 - line 33 page 698; figure 1a page 703; figure 7a	1-73
A	ALEXANDER JANET E ET AL: "Amino acid changes affecting the activity of pneumolysin alter the behaviour of pneumococci in pneumonia" MICROBIAL PATHOGENESIS, vol. 24, no. 3, March 1998 (1998-03), pages 167-174, XP002341460 ISSN: 0882-4010 the whole document	1-73
A	LOCK ROBERT A ET AL: "Sequence variation in the Streptococcus pneumoniae pneumolysin gene affecting haemolytic activity and electrophoretic mobility of the toxin" MICROBIAL PATHOGENESIS, vol. 21, no. 2, 1996, pages 71-83, XP002341461 ISSN: 0882-4010 the whole document	1-73
A	WO 90/06951 A (PATON, JAMES, CLELAND; HANSMAN, DAVID, JOHN; BOULNOIS, GRAHAM, JOHN; A) 28 June 1990 (1990-06-28) the whole document	1-73

INTERNATIONAL SEARCH REPORT

ɔnal application No.
PCT/GB2005/001774

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 67,68
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 67 and 68 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

ion on patent family members

Intern pplication No
PCT/GB2005/001774

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2001014332 A1	16-08-2001	US 2005070695 A1	31-03-2005
		AU 740956 B2	15-11-2001
		AU 8407898 A	10-02-1999
		CA 2297374 A1	28-01-1999
		EP 0998557 A2	10-05-2000
		HU 0002475 A2	28-10-2000
		JP 2001510031 T	31-07-2001
		NO 20000257 A	21-03-2000
		WO 9903884 A2	28-01-1999
WO 9006951 A	28-06-1990	AT 205534 T	15-09-2001
		AU 626961 B2	13-08-1992
		AU 4756490 A	10-07-1990
		WO 9006951 A1	28-06-1990
		BG 51358 A3	15-04-1993
		CA 2005704 A1	16-06-1990
		DE 68929323 D1	18-10-2001
		DE 68929323 T2	18-04-2002
		DK 115591 A	14-08-1991
		EP 0449856 A1	09-10-1991
		ES 2160570 T3	16-11-2001
		FI 103122 B1	30-04-1999
		HU 58804 A2	30-03-1992
		JP 4503948 T	16-07-1992
		JP 3237842 B2	10-12-2001
		MC 2178 A	22-05-1992
		NO 912226 A	25-07-1991
		RU 2121481 C1	10-11-1998
		US 6716432 B1	06-04-2004